AD A043021

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1 JULY 1975 - 30 SEPTEMBER 1976

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U.S. ARMY
MEDICAL RESEARCH AND DEVELOPMENT REPORT
RCS MEDDH-288 (RI)

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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER RCS-MEDDH-288 (R1)	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Army Medical Research & Development Technical Report.		5. TYPE OF REPORT & PERIOD COVERED Annual progress rept. 1 July 1975-30 September 1976
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR Gunter F. Bahr, Joseph A. Boccia, J. M. Ballo, H. W. Casey W. T. Hockmeyer		8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS Armed Forces Institute of Pathology Washington, D. C. 20306		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
11. CONTROLLING OFFICE NAME AND ADDRESS Army Medical Research & Development Command Washington, D. C. 20314		12. REPORT DATE 1 October 1976
		13. NUMBER OF PAGES 73
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)		15. SECURITY CLASS. (of this report)
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

3E762720A835; 3A762758A819

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Cytologic water purity assay	Cytologic images	Occupant kinematics	Food
Quantitative cyto-morphology	Cytologic pattern recognition	CALSPAN three dimension program	Radiation Sterilization
	Computer simulation model		Beef
	Aircraft accidents		Experimental

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The research projects reported in this Progress Report are as follows:
Project: Environmental Quality Protection   (3E762720A835)
Project: Army Aviation Medicine   (3A762758A819)
Project: Tropical Medicine   (3A762759A831)
Project: Lesions in Animals Fed Enzyme   (1J664713D47)
        Inactivated Frozen & Irradiated Beef

DD FORM 1473   EDITION OF 1 NOV 65 IS OBSOLETE

Immunity
Plasmodium berghei
Sporozoite
Mice

U.S. ARMY RESEARCH AND DEVELOPMENT

TECHNICAL REPORT

RCS-MEDDH-288 (R1)

ARMED FORCES INSTITUTE OF PATHOLOGY

WASHINGTON, D. C.   20306

ANNUAL PROGRESS REPORT

1 JULY 1975 - 30 SEPTEMBER 1976

ACCESSION for
NTIS   White Section ☑
ODC   Buff Section ☐
UANNOUNCED   ☐
S I ICATION

DISTRIBUTION/AVAILABILITY CODES

A

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Research Supported By:

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
WASHINGTON, D. C.

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RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY				1. AGENCY ACCESSION* DA OA 6613	2. DATE OF SUMMARY* 76 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636
3. DATE PREV SUM'RY 75 10 01	4. KIND OF SUMMARY C. Change	5. SUMMARY SCTY* U	6. WORK SECURITY* U	7. REGRADING*	8a. DISB'N INSTR'N NL	8b. SPECIFIC DATA-CONTRACTOR ACCESS ☒ YES ☐ NO	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	6.27.20.A	3E762720A835	00	150
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS 114F			

11. TITLE (Precede with Security Classification Code)*
(U) Analysis of Cytotoxic Reactions Produced by MUST-Water Constituents

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*
007800 Hygiene and Sanit., 005900 Environmental Bio., 009800 Med. & Hosp. Eq.

13. START DATE 7410	14. ESTIMATED COMPLETION DATE CONT.	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
a. DATES/EFFECTIVE:	EXPIRATION:	PRECEDING 76		
b. NUMBER:*		FISCAL YEAR		
c. TYPE:	d. AMOUNT:	CURRENT 7T	1.2	11
e. KIND OF AWARD:	f. CUM. AMT.			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
NAME:* Armed Forces Institute of Pathology ADDRESS:* Washington, D. C. 20306	NAME:* Armed Forces Institute of Pathology ADDRESS:* Washington, D. C. 20306
RESPONSIBLE INDIVIDUAL Hansen, J. L., COL, MC, USA NAME: 202-576-2905 TELEPHONE:	PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution) NAME:* Bahr, G.F., M.D. TELEPHONE: 202-576-2915 SOCIAL SECURITY ACCOUNT NUMBER:
21. GENERAL USE	ASSOCIATE INVESTIGATORS NAME: J. A. Boccia, LTC, MC, USA NAME:

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Cytologic Water Purity Assay; (U) Quantitative Cyto-morphology; (U) Analysis of Cytologic Images; (U) Cytologic Pattern Recognition

23. TECHNICAL OBJECTIVE,* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Develop cytoassay for detection of MUST-water impurities through extracting quantitative morphologic descriptors from digital images of cells cultured in waters tested. Method should extend to general water testing. At close of CY76, US Army to possess minicomputer system and protocols for water toxicity cytoassay. Implication: reduce need for animal toxicologic testing. Desired result: higher and more quantitative data rates: lower costs; simpler laboratory logistics. Application to MUST re-use water primary; extension to water testing where military presence is possible, probable, or actual within applicability. Military-industrial effluent cytoassay an important consideration.

24. (U) Purchase upgrade for minicomputer hardware and software. Upgrade image digitizing light microscope subsystem. Design and fabricate (D&F) electronics for interfacing/ control of scanner to/by minicomputer. Generate application specific programs. Standardize culture and staining procedures. Run feasibility experiments with raw water and water with known toxic additives; technical problems; standardize culture in presence of toxicants/raw water; theoretically develop and implement quantitative estimator of staining variability in contrasts and intensity; pre-analysis validation of image data; extract cell image descriptors of cytopathologic value which sensitive to exposure time and toxicant concentration; production of adequate experimental throughout on modest minicomputer system. Decelerating factors; procurement delays and defects in new equipment and software. Accelerating factor: addition of 1 man-year/year of profession-al effort, starting FY75.

25. (U) 7507-7609. Electronic interfacing and testing of equipment added to upgrade scanning microdensitometry system completed. Program subsystem for interactive

*Available to contractors upon originator's approval.

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DD FORM 1498
1 MAR 68

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Title: (U) Analysis of Cytotoxic Reactions   Project No. 3E762720A835
          Produced by MUST-Water Constituents

acquisition of digital test cell images 90% completed. This subsystem
allows acquisition of test cell images without prior standardization of cell
preparatory techniques. Cell image data is recorded in format appropriate
for input to analytic subsystem software which will perform image measurements
and statistical analysis. Subsequent to acquisition subsystem completion,
feasibility of particular cell image measurements in the discrimination of
cytoxicity will be studied. Data pertinent to the development of production
system will also be acquired.

ANNUAL PROGRESS REPORT

TITLE PAGE

Project No. 3E762720A835   Title: Analysis of Cytotoxic Reactions
                                        Produced by MUST-Water Constituents
Task No.   00

Name and Address of Reporting Installation:

  Armed Forces Institute of Pathology
  Washington, D. C. 20306

Name of Department: Cellular Pathology

Period Covered by Report: 1 July 1975 - 30 September 1976

Professional Authors: Gunter F. Bahr, M.D.
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Report Control Symbol: RCS-MEDDH-288(R1)

Security Classification: Unclassified

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BODY OF REPORT

Project No.   3E762720A835

Task No.   00

Title: Analysis of Cytotoxic Reactions
       Produced by MUST-Water
       Constituents

SUMMARY

This annual interim report describes progress in the construction of a
computer assisted scanning light microscope research system for the acquisition
of digital cell images. The microscope system is for use in the development of
a cytologic assay for water toxicity. It will be used to assess the feasibility
of using cells in culture for the detection of noxious substances in water
supplies. In the event of feasibility, parameters which effect the construction
of field implementable systems and the performance of toxicity testing with such
systems is to be assessed. The research system being developed as part of this
project is intended as an R&D resource and is not designed for production
environments or field use.

The work described in this report has principally to do with the development
of hardware interfacing of microscope equipment to a small in-laboratory mini-
computer, the development of a computer program system to enable the acquisition
of cell images for subsequent analysis, and morphologic investigation at the
light and electron microscopic levels(both TEM and SEM) of cellular effects
resulting from exposure to water samples containing model toxicant (2,4-
dinitrophenylhydrazine and RO permeate). The latter investigation is performed
to obtain evidence of cellular alterations which might serve as sensitive
indicators of toxicity when measured in digitized cellular images.

These developments are part of the objectives for which the background
is given in (1).

At this writing, the scanning microdensitometer system is nearing a state
of completion where experimental cell image data acquisition can begin. At
such time, the analytic phase of the project shall commence.

Parallel to system development, light microscopic and transmission and
scanning electron microscopic investigations have indicated that cell morphologic
alterations occur in Mouse L-cells exposed in culture to 2,4-dinitrophynyl-
hydrazine (DNPH) and RO permeate-these agents being used as model toxicants.
Cells exposed for 1 to 4 days to varying concentrations of toxicant exhibited
consistent and graded response to both agents. Effects of RO permeate, in the
concentrations used, were more pronounced than those for DNPH.

Both the frequency and intensity of cytomorphologic change exhibited by
members of the cellular population exposed to these model toxicants appears to
vary directly with concentration and duration of exposure. Mouse L-cells,
normally stellate or fusiform in configuration, become more rounded with greater

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frequency as dose and exposure increase. Similarly, the normally villous cell surface becomes progressively less villous with the development of local blebing from the plasma membrane. The ovoid nuclei seen in unexposed cells (controls) rounds and becomes relatively hyperchromatinic. Overall cell size diminishes. Such changes are observable in significant numbers of cells exposed in culture for one day. These findings are accumulated on the basis of light microscopic examination of Giemsa and Papanicoloau stained cells and scanning electron microscopic examination of cell surface structure.

Similar cytological observations obtain using the light microscope and the Feulgen reaction with Napthyl Yellow cytoplasmic counterstain. Trial scans with Geimsa stain suggest too little nucleocytoplasmic contrast for microdensitometry.

Because of these observations, the cellular features which promise the most likely indication of early intoxication are those which measure 2-dimensional cytoplasmic and nuclear texture, cytoplasmic and nuclear optical mass, cell and nuclear shape, and nucleocytoplasmic size ratio. Examples of such features are: rate of variation of nuclear and cytoplasmic density per square micron of area, total nuclear and cytoplasmic density, radial distance from nuclear or cytoplasmic centroid to the nuclear or cytoplasmic border as a function of angle of revolution about the centroid (geometric center), nuclear area, and cell area.

Features of the above type will be measured first during the analytic phase of this project. This list is not intended to be exhaustive and others will probably be added based upon trial of the ones listed. Further, compounded features, consisting of arithmetic combinations of more primitive ones will also be utilized.

The prominent response of cells exposed to toxicants for one day makes reduction of culture time possible. Experiments involving exposure in the range of 0 - 24 hours are planned. Since changes after short exposure or to small amounts of toxicant may not be visible to the eye, the scanning micro-densitometer is needed to produce computer pictures in which early consistent, non-visable detectably graded alterations may occur. The graded and prominent response obtained to date is consistent with, but not proof of this possibility. This proof is of major importance in this project and one of several primary applications for the scanning microdensitometry system in the development of a cytologic method for the certification of water.

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SYSTEM HARDWARE

The following discussion makes use of Figure 1 which is a schematic diagram depicting the major system hardware components and their interrelationship. Reference numbers given in this section point to the corresponding hardware component in Figure 1. A legend giving the names corresponding to the component numbers is provided in the figure.

The cellular specimen (1) is mounted on the motorized stepping stage (2) manufactured by Karl Zeiss. The test cells are grown on 8 X 22 mm glass cover-slips in Leighton tubes. When removed from culture the cells are fixed on the coverslips using 95% methanol. After appropriate staining, the coverslips are permanently mounted on ordinary 7.4 X 2.5 cm glass microscope slides.

The stage (2) is capable of stepping rates to a maximum of 200 steps/second and the stepping distance is 0.5 micron/step. It is interfaced to a small in-laboratory minicomputer manufactured by Interdata (5). The interface was designed and fabricated in this laboratory's electronics shop and is completely functional. It permits computer controlled movement of the stage.

A research quality light microscope (3), with built-in photomicrographic capability (35mm), is used for both human and computer visualization of the specimen. The microscope's stand, body tube, and all optics are manufactured by Karl Zeiss. The usual working objectives for actual scanning of selected cells are 100X, NA 1.32 and 63X, NA 1.40 oil immersion brightfield lenses. The coverslip size is small enough so frequent oil application during cell selection and scanning is not required. Focusing is manually performed. Visual examination of the specimen is performed using 10X or 12X eyepieces which are equipped with graduated crosshairs. The microscope tube magnification factor for purposes of computer image digitization is 10X. Filtered white light from a tungsten-halogen source is used for transillumination. The filtering bandwidth and transmission maximum is varied according to experimental staining conditions.

A photomultiplier tube (4) is mounted on the microscope and used as image transducer. The tube was manufactured by EMI and has a signal to noise ratio of greater than 100:1. The analogue output of the photomultiplier tube is A/D converted using components of a Zeiss UP-1 Cytoscan which has been adapted for application to this project and interfaced to the minicomputer (5). This interface was designed and fabricated in the laboratory's electronic workshop and is completely functional. Through the interface, optical density measurements may be commanded under computer control and resulting data transmitted to the computer. The maximum data rate is 60 pixel measurements/sec.

Two joystick controls for stage movement are available (7,8). One is an off-line control (8) which causes direct movement of the stepping stage without intervening computer control. In conjunction with manual stage movement controls (thumbwheels) this joystick is used to perform initial alignment of the specimen after mounting on the stage. In this alignment, reference marks (See Figure 2) previously impressed into the glass slide and the eyepiece crosshairs' intersection

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are made to coincide. The marks are first found by coarse movement with the stage thumbwheels. Then, under oil at 1000X magnification, the off-line joystick (8) is used to move the crosshairs to coincidence with the selected slide reference mark- of which there are four. This relatively fine adjustment must be made with hands off the stage and using the stepping motors. (See Figure 3.) What appears to be good alignment with hands on the thumbwheels reverts to misalignment when the thumbwheels are released because of stage movement upon withdrawing even this small amount of pressure.

Off-line stage movement controls (thumbwheels and joystick) are used only during slide alignment. Thereafter, only on-line controls are used for stage movement. The essential difference between on-line and off-line movement is that: a.) during on-line movement the computer drives the stage, and b.) the position of the stage with reference to the initial alignment is tracked. The initial position is taken as the origin, (0,0), in a two dimensional Cartesion coordinate system. Distance is measured in millimeters expressed to three postdecimal significant figures (nearest micron). Selected cells may be located by recording the co-ordinates and mark number which obtain when the crosshairs are placed upon the cell. This permits refinding the cell for any required purpose-such as scanning preselected cells which were not scanned, rescanning cells, and photomicrography.

The on-line digital joystick (7) was manufactured by Measurement Systems, and is used by the scan operator to freely move the specimen when searching and selecting cells for scanning. Using this joystick, stage motion is tracked by the computer. The stick output is digital and its interface to the computer was designed and fabricated in the electronics workshop of the project laboratory. It is completely functional.

Unlike the off-line joystick (8), which is sensitive to stick displacement, the on-line stick is sensitive to pressure and does not move when toggled (isometric stick). This renders it relatively sensitive with rapid stage response to the application and removal of pressure. Both the direction and speed of motion may be controlled through its use. The on-line stick, therefore, acts both as a rudder and throttle. It has been selected for this project, not only for its use in searching the specimen, but also for the role it plays in certain editing operations on the cell image which this system permits and the project strategy requires. Editing involves tracing various cellular substructural boundaries using the on-line stick and crosshairs' intersection as a cursor. Because the on-line stick is a pressure transducer, stage response is fairly immediate. The actual working sensitivity may be adjusted by the operator during editing and searching. This capability is provided in software and is not built into the joystick itself.

As ASR-33 Teletype (6) is used as the scan operator's console. Through the keyboard the operator may supply parametric data to be associated with one or more cell images and give scanning subsystem commands. The operator receives

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instructions (e.g., command prompts) and messages from the subsystem on the Teletype printer. In particular, stage motion in meandering patterns or to specific co-ordinates may be commanded from the Teletype keyboard. As such, the Teletype (6) is also an on-line stage control. Any motion caused by command from this unit is tracked by the computer.

A small computer disc (10), manufactured by Diablo for Interdata, is used to temporarily store all data connected with each scanned cell. The disc also stores data required for the control of the scanning process. This includes the actual programs implementing the Scanning Subsystem and many parameters which are frequently updated in the course of scanning operations and describe at any point in time the state of the system. An example of such control parameters is the current stage coordinate location and there are over 200 such parameters involved in subsystem control. The disc is required for these various purposes because direct access techniques must be used in the performance of computer input/output connected with scanning. Due to the number and size of the programs involved, the amount of data being acquired, and the small amount of memory available in the computer, extensive use of auxiliary storage is made. Sequential access, even to and from very fast magnetic tape drives, would be impractical.

After all data connected with a particular cell is acquired and stored on disc, it is written on the operator's command to 9 track, industry compatible magnetic tape (9). The tape drives are manufactured by Kennedy and the tape drive controller and interface by Xebec Systems. The 10 million bytes of disc storage are insufficient to store the digital cell image, its associated parametric data, and any editing trace data generated for large numbers of cells. The disc must also store control data and system programs. Permanent storage of cell data is therefore accomplished with magnetic tape.

For display of halftone cell images, parametric data listing, and line drawings (e.g., trace boundaries and data plots), a printer/plotter (11) is available. This device was manufactured by Gould and is electrostatic in type. Printouts and plots are rendered on 8.5 x 11 inch fanfold paper. This size was selected for convenience of storage in ordinary file folders, cabinets, and loose leaf binders. Further, the non-impact technology reduces noise pollution in the laboratory environment. This is a significant cause of fatigue and distraction for laboratory personnel and impact printers can generate a considerable clatter.

The hardware system components, as depicted in Figure 1 and described in this section are installed and fully operative. From the hardware point of view, the system is complete. As configured, the system serves both data acquisition and analysis. When data acquisition is not taking place, the computer, disc, magnetic tape drives, printer/plotter, and typewriter console are used for analysis and program development. The scanning microdensitometric components are powered down.

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SYSTEM SOFTWARE

The overall system design includes software support which can be classified into seven (7) major categories. These are outlined in Table 1. The general purpose software in categories 1 and 2 is supplied by the minicomputer manufacturer- with the exceptions of special driver program extensions described in the next paragraph. The remainder of the software support for the system is application specific and is developed by project personnel.

The programs in Category 2 of Table I are all supplied by the manufacturer and support the functions of program development and execution. All programs written for this project are implemented in Interdata, Inc.,FORTRAN-V, Level II and/or COMMON ASSEMBLER LANGUAGE. This FORTRAN language is a superset of ANSI X3.9-1966 FORTRAN. Among other extensions, in-line assembler language code is permitted. Since this capability is particularly suitable for programming the control of on-line instrumentation, some of the application programs in categories 3 and 4 are written in both Interdata's FORTRAN and COMMON ASSEMBLER LANGUAGE.

Category 3, Table I consists of the Scanning Subsystem. This collection of computer programs forms the interface between the scan operator and the rest of the system software and hardware during the process of image acquisition. Various functions required during each image scanning session are provided by this Subsystem. Included are facilities for the staged entry of operator supplied data which is to be associated with each image scanned. Data associated with multiple cell images need be entered only once and is automatically recorded with each digital cell image.

The Scanning Subsystem also enables systematic traverse of the microscopic slide in the form of an automatic meander pattern which may be stopped at any desired position by the operator. Joystick controlled movement is also permitted and the two methods of searching for cells can be utilized interchangeably.

During any searching operation cells may be named and their locations, expressed in millimeters to three significant postdecimal figures (nearest micron), can be automatically recorded. Cells may also be selected from randomly placed microscope fields. Such field placement is performed by selection of co-ordinates from a bivariate uniform distribution over a rectangular search zone whose dimensions and position may be defined by the operator. However cell locations are selected, the co-ordinates are automatically recorded upon appropriate operator command.

Once positioned at a particular cell which has been selected for scanning, the operator may pre-edit the scene observed through the microscope eye-pieces. Virtual microdissection of the cell and separation from its neighbors may be performed by tracking paths and placing point locators around, between,

and on significant cellular features. This is done with the digital joy-
stick and microscope eyepiece crosshairs used as a cursor. The scene is
essentially annotated by recording the track trajectories and point locations
through which the cursor passes. This graphic editing data is eventually
recorded on magnetic tape along with the actual digital cell image and its
associated parametric data.

The editing function permits significant cellular structures of minimum
dimension no less than 0.5 microns to be located subject only to the condition
that the operator can see them. Prior dependence upon particular staining
properties for the location of structures is avoided, since these properties
are among the objectives of the project investigation. Examples of cell
structure location and separation from neighboring cells which are permitted
by the editing function in the Scanning Subsystem are: cell and nuclear border
trajectories, nucleolar position pointers, and delineation of scanning
perimeter. The latter is a closed, non-intersecting curve around the cell of
interest. When the cell is scanned, only the contents interior to the curve
or on it are digitized and recorded as part of the computer picture. Picture
elements (pixels) in the digital raster constituting the computer picture which
lie outside the scan perimeter are automatically set to backround. This
eliminates the majority of unwanted neighboring detail even before the cell is
scanned. It also reduces the scanning time by confining scanning steps to the
area of interest.

The actual physical scanning of a selected cell is performed after
accomplishing any editing necessary. At a minimum, a scan perimeter must be
traced to separate the cell from unwanted neighboring detail. Additional
editing for location of cellular structures is optional. The details of this
optional editing may be preselected by project supervisory personnel from a
library of editing procedures, each of which is designed to permit graphical
annotation of particular cellular substructure. The library of editing
procedures may be altered by additions and deletions. In addition to the scan
perimeter, the subsystem requests the performance of each editing procedure
which has been preselected from the library. Scanning itself is not permitted
unless these procedures are completed. This insures that the interactive image
annotation required is performed by the scan operator- who performs the editing
but cannot control its nature and extent.

Once the required editing is completed, physical scanning of the cell
may be initiated by appropriate operator command. The cell is scanned and its
digital image recorded. To this point, all recorded data, be it associated
parametric data entered by the operator, graphic image editing data, or the
actual digital cell image, is placed in computer disc files allocated for the
purpose. This technique is applied in order to permit random access to
auxiliary storage of the data- a requirement for the kind of computer operations
performed by the scanning subsystem. When cell scanning is complete, the

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operator may cause all recorded data for the cell, regardless of type, to
be recorded on magnetic tape. A format for such tape recording has been
devised and a preliminary draft of the specifications transmitted to USAMRDC
as an appendix to the interim report (2) covering the period 1 May - 31 Aug 1976.

In this manner, all data pertinent to a given cell image is recorded
with that image and is never separated by the system. When subsequent sorting
of cell image collections is performed, all data moves with its image and is
therefore readily available for participation in any image processing to
be done.

The feature extraction function in the Scanning Subsystem will allow
the extraction of operator selected features from the cell image immediately
after scanning. This provision is made for exploratory and program development
purposes only. Normally, feature extraction will be performed in batch mode
on multiple cellular images by the Analytic Subsystem's Feature Extraction
Division programs. This program division of the Analytic Subsystem is open
ended in the sense that it may be continually added as required. It is never
"completed." The only purpose of feature extraction during scanning sessions
is immediate assessment of values for particular test conditions and the
debugging of feature extraction programs.

Of the eight (8) program units listed in Table I for the Scanning Subsystem,
the Feature Extraction Program simply accesses routines in the Analytic Subsystem
and, therefore, grows with this subsystem. At this writing, the Image Edit
Program is nearly complete and the Scan Program is still to be coded. All
other program units are completely functional.

The Analytic Subsystem, Category 5, is to be the object of development
in the immediate future. As previously mentioned, the Feature Extraction
Division of programs is open ended. The same is true for the Statistical Division.
This Subsystem's function is to analyse the data captured during scanning.
Collections of cellular images must be recorded in such a manner that repeated
analysis involving the extraction of various batteries of features and
statistical techniques is possible. The content and format of captured image
data on magnetic tape has been designed for this purpose.

Category 6, Table I is the Display Subsystem. This category is also
open ended and developed in correlation with the Analytic Subsystem. Its
purpose is to print hard copy halftone cell images, parametric data listings,
and graphical line drawing which display image editing and statistical analysis
results. As such, it is added to as needed and in parallel development with
the Analytic Subsystem of software.

Category 7, the System Subroutine Library is a collection of FORTRAN
callable subroutines which perform frequently utilized computations in various
parts of the system. This library currently numbers approximately 60 subroutines-
all of which are project implemented. The system subroutine library is open
ended.

Category 4, the Utility Subsystem, is a collection of programs which are used by designated individuals to perform certain technical operations. These operations may apply to any other subsystem. Due to their specialized and technical nature, further details will be given in the sections of this report dedicated to the functions of the system which these programs support.

SCANNING SUBSYSTEM OPERATIONS

This system is being constructed to obtain data particularly suited to the development of a cytoassay technique for water purity. Various aspects of its operation deserve comment in the light of this objective.

Accurate image analysis is not possible without a sufficient number of good digital images for each experimental cell population. Not only has previously existing scanning equipment been upgraded by a speed factor of 3-5 times, but real time data checking has been included to assure data quality in digital images. Every pixel measurement in the digitization process is subject to range testing. Should any particular measurement attempt result in spurious image data, the measurement is retried. Data is not recorded unless it is valid. In off-line recording, as done previously, invalid data was detected at the time of analysis. Such after-the-fact checking can result in entire collections of images being inadequate for use due to unnoticed malfunction. This does not happen with the system constructed for this project, since an image does not get to magnetic tape unless it can pass various data checks. This is particularly important for the project, since larger numbers of cell images are involved than previously and, therefore, more images and manhours would be at risk.

The system is capable of being operated by non-computer oriented personnel. Typically, personnel with background in cytotechnology and/or histotechnology can be employed as scan operators. Certain scan control data items can be entered into the system to exert control over the data acquisition process. Supervisors need not be immediately available during periods of data capture, since their instructions are incorporated into the scanning process in the form of control data.

A particular example of this is the proper recording of adjunct parametric data not obtainable by computations performed on the digital image itself. Examples are: various experimental conditions such as staining and fixatives used, hardware calibration conditions, hardware control settings, and scan operator identification. Such data is necessary for different purposes during subsequent analysis.

The amount and nature of such parametric data is determined by project supervisors. This is done by defining the contents of various data directory files using utility software in Category 4 of Table I. Specifically, the Data Directory Editor and Data Subdirectory Creator programs are used to determine the content of directory and subdirectory disc files. These directory files describe and point to items in data files which must be filled by the scan operator in the course of a scanning session. No additional programming is required to incorporate or delete new parametric data items-only the above programs need to be used. The parametric data base is, therefore, expandable with minimum effort.

The sequence actions taken during scanning by the operator is controlled by the system through the process of command prompt - response interaction. The operator enters nothing into the system without prior request by the system. If any command or data entry is invalid, the system rejects the input with appropriate error message and forces a retry by the operator. Operators need not memorize command and data entry sequences, since the order of these actions is controlled by the system. Generally speaking, failure of the operator to perform certain data entry and hardware initiation tasks will result in rejection of any attempt to proceed. For example, failure to completely pre-edit a cell before scanning results in rejection of any attempt to scan it. In this way, data associated with each cell image is assured complete and valid.

On the other hand, data which remains constant for multiple cells need not be entered repeatedly. The system identifies data which must change for each cell (e.g., cell name and location) and forces new data entries for these changing parameters upon the selectionof each new cell. Data which does not change from cell to cell is altered at the option of the operator. Illumination wavelength, pixel measurement replications, and scan operator identification are examples of the latter.

A master directory - subdirectory approach has been used which takes into consideration the exploratory nature of the project. Experimental conditions will vary from time to time. This variation will alter the nature and values for associated parametric data. Master directory creation for this data is performed using the Data Directory Editor program in Category-4. However, not all data parameters defined in the master directory need be used. A subdirectory may be created which consists of a subset of those items in the master. The Data Subdirectory Creator program in Category 4 is used for this purpose. During scanning, the subdirectories are used to guide the operator through data entries.

All parametric data is entered/changed in accordance with a staging scheme. The staging corresponds to certain of the phases in the scanning process. The scanning phases are: LOGIN, INITIALIZE, SEARCH, EDIT, SCAN, EXTRACT, LOGOUT, and OPTION. During each of these phases the operator can and must perform appropriate actions which together constitute the acquisition of cellular images.

A. OPTION Phase

The OPTION phase offers the operator the opportunity to select the next phase which is to be entered. This is the first phase entered when beginning a scanning session. When the OPTION phase is entered at the beginning of a scanning session, the operators must next enter the LOGIN phase. The option phase utilizes system control data in a special file kept on magnetic disc to determine when a scanning session is begun. This file contains many system

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control data items, among which are the last phase to execute and its termination status (e.g., successful normal termination or premature termination). The OPTION phase offers the operator an opportunity, through command prompts, to select one from a permissible list of next phase selections. The only phase selectable after a previous LOGOUT phase completion is LOGIN.

B. <u>LOGIN Phase</u>

During the LOGIN phase, the operator enters data which is to remain unchanged for the entire scanning session. Much of the required data may still be valid from the previous session. If this is the case, only a small portion of the LOGIN data subset need be entered by the operator. Examples are: the operator's identification, and current date. Data which does not change from session to session is alterable at the operator's option. All data recorded during this stage may be listed to the operator's console (printer). Exit from the LOGIN phase is to the OPTION phase.

C. <u>INITIALIZE Phase</u>

After successful execution of the LOGIN phase, OPTION permits the selection of only the INITIALIZE phase. During INITIALIZE, scanning hardware switches are set, the microscope slide is mounted and slide mark alignment is performed. The scanner hardware may be tested with dummy scans. Various data items pertaining to scanner switch settings, illumination, slide identification, and scanning conditions are enterable at this point. If this phase is entered from OPTION after a previous LOGIN, then data items which change from session to session must be reentered. Other items are entered at the operator's option. The INITIALIZE phase is enterable from OPTION during a scanning session from phases other than LOGIN. In this case, all data entry is optional. That is, the operator may change specifically the data items for which reentry was selected. All data entered during this phase may be listed to the operator's console. Exit from INITIALIZE is to the OPTION phase.

Previous mention has been made of a special system control file. This file contains information concerning the state of the system at any point in time. Its data are not recorded with the cell images, since they do not apply to the image but rather to the system. In addition to providing system control, the control file allows recovery from system failures. Should power or machine failure occur during a session, the operator can return to the last condition prior to failure. This is of particular importance with reference to co-ordinate position on the specimen slide. At most, the last attempted cell scan is at risk. All previously scanned cells and associated data are recorded on magnetic tape after successful scanning. The operator is provided with the capability of returning to the point of failure and continuing. If malfunction occurred during the scanning of a particular cell, it is rescanned.

The mechanism employed by the Scanning Subsystem is to update the control file at certain milestones during the session. Power failure and the majority of machine malfunctions do not alter data in the control file on magnetic disc.

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Neither does operator error destroy this data. It thus becomes unlikely
that much data will have to be acquired twice due to loss as a consequence
of malfunction. The amount of data required by this project is large enough
to require this type of protection. Loss of large numbers of images because
of occasional malfunction would severely compromise scanning throughput.

During the INITIALIZE phase only, the operator may cause the contents
of the control file to be printed on the console. This provides a means of
viewing the state of the system when desired and greatly aids in the process
of recovery when needed.

The INITIALIZE phase may be entered after the LOGIN and SEARCH phases
only. Exit from the INITIALIZE phase is to the OPTION phase. When the LOGOUT
phase is executed, certain data INITIALIZE phase items which change from session
to session are effectively erased. These must be reentered when INITIALIZE is
next executed.

D. SEARCH Phase

The SEARCH phase permits perusal of the specimen slide, the movement to
specific slide co-ordinates, and the recording of cell locations with
designation of cell name. Facilities which promote proper selection of cells
and valid sampling from the parent cell population are also included.

The slide may be searched in an automatic meander pattern or by joystick
control. In the meander method, the operator may specify (in millimeters) the
dimensions of a rectangular search zone. Upon command, the stage will be moved
in a meander pattern within the limits of the defined zone. The motion may be
stopped at any time by the operator. When a zone is completely covered, the
operator may displace the zone in one of eight directions (See Figure 4). In
this manner it is possible to cover an entire coverslip in one or more
adjacently placed search zones, and cover any particular zone as many times
as desired. In order to evenly distribute wear on the stage motors, the major
axis of meander is alternated for each search zone traversal. The major axis
of meander is the axis in which the majority of stepping takes place. The
minor axis of meander is traversed but once in meandering over the search zone
(See Figure 5). This alternation is provided automatically by the subsystem
software and no operator action is possible or necessary. Past experience
without such alternation indicates excessive wear to one motor with decreased
mean time between failure.

The automatic meander may be stopped at any time. The operator may
optionally choose to utilize the on-line joystick or some other command in the
search phase. In any case, the meander search may be continued from the point
of cessation. When a command to do so is issued, the stage is first returned
to the point where the meander was stopped and the zone search is continued.
In particular, this allows the alternating use of meander search and on-line
joystick. The meander is used to search and find cells. The joystick is used

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to go to the cell, edit it, and after scanning, return to the meander search where it was left. Thus a systematic and comprehensive search and selection of cells is possible.

Movement to specified co-ordinate positions is referred to as seeking. Seeking differs from searching because, in the former, a target destination is specified, whereas in the latter case, whether by automatic meander or joystick, stopping points are not previously specified. Target co-ordinates for seeking operations are supplied from various sources, depending upon the situation. For example, when cells are scanned, their co-ordinates are recorded and parametric data listings show them. If it is desired to refind the cell for any reason, the slide may be mounted, aligned on the appropriate reference mark (also recorded and listed), and the specific co-ordinates found through the use of a seek command in which the co-ordinates are given by the operator.

Other seeking functions include: automatic return to the reference mark (origin), absolute or relative displacement from the current slide position to operator specified co-ordinates, passage to the next co-ordinate in a pre-picked list of co-ordinates, and movement to the next co-ordinate in a list of co-ordinates pseudorandomly generated from a uniform bivariate distribution over a rectangular zone of predefined dimensions and position.

Return to the origin is frequently needed prior to reinitialization using the INITIALIZE phase. This function is also critical if recovery from subsystem malfunction is required. Since milestone co-ordinates are kept and periodically updated, finding the last milestone prior to failure is possible by a three step procedure: 1) restart the subsystem, 2) realign the micro-scope slide on the appropriate reference mark, and 3) issue a special seeking command which returns the stage to the last recorded milestone in the control file.

Movement to absolute or relative (in millimeters) co-ordinates is frequently used for positioning to preselected locations and to estimate coverslip and search zone dimensions. Particularly in the case of relative seeking displacements, the breadth and width of usable coverslip area may be determined. This in turn allows judicious choice of search zone dimensions for automatic meander. For example, knowing the dimensions of usable coverslip allows the selection of search zone dimensions such that the usable area is covered by one or more adjacent zones with minimum overlap of unusable areas.

By using any of the mechanisms available for searching the coverslip, cells may be preselected by causing their locations to be recorded in a control file list. The cell location is defined by placing the eyepiece crosshairs unabiguously over the cell in question- that is, on the cell desired and on no other. On command, the current co-ordinate position is recorded in the next available position in the aforementioned list. Special seek commands are

available which allow movement from any position to any co-ordinate location recorded in the list. Also available is the capability of moving to each listed position in the order of list occurrence. An existing list may be either erased or cut down to a specified number of entries. Maximum list capacity is 50 pre-picked locations. One of the major utilities of this function is to allow persons other than the scan operator to select cells for subsequent scanning.

A function for the pseudorandom generation of from 1 to 100 co-ordinate locations has been built into the scanning subsystem in order to promote the representative selection of cells for scanning. Sampling technique is critical for the proposed cytoassay technique, since the toxicity of any water sample is to be measured by its quantitative cytopathologic effect on the exposed cell population. This parent population consists of all the cells on a given coverslip. Each such coverslip contains cells which have been exposed for a particular period of time to a given concentration (titer) of raw sample water.

Only a sample of cells from each coverslip are to be selected for scanning and analysis. It is necessary that this sample be selected in an unbiased manner. The visual quality of a cell must not be allowed to influence the decision to select it or not. The pseudorandom generation of co-ordinates is used in one of two ways to promote unbiased selection: 1) by generation of co-ordinates from which the nearest non-mitotic cell to the eyepiece crosshairs is selected, or 2) by generation of co-ordinates which serve as the start point (See Figure 4) for search zones from which all non-mitotic cells hit during meander are selected. For operational purposes, non-mitotic means not visably in mitosis. When method (1) is used, and when two or more cells are apparently equidistant from the graduated eyepiece crosshairs, the cell lying at the least clockface position (1-12) is chosen. Using method (2), all non-mitotic cells hit by the eyepiece crosshairs during meander search of the rectangular zone whose upper left hand corner is the random co-ordinate pair are selected. Method (1) shall be called random cell selection and method (2) random zone selection.

When generated, random co-ordinates are recorded in a control file list which may be printed to the operator's console on command. Similar to seeking operations for co-ordinates in the pre-picked list, the facility to seek listed co-ordinates in both arbitrary and sequential list order are included in the Scanning Subsystem. This means that, in the case of random cell selection, the qualifying cells may be sought and scanned in arbitrary or list sequential order. The latter order insures complete selection without omission of any co-ordinate location. In the case of random zone selection, similar statements apply to the search of all zones whose upper left corner is represented among the random co-ordinates listed.

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It should be noted that the pre-picking and random co-ordinate generation functions are designed to be used in concert. Random co-ordinates can be generated first. Then using either the random cell or random zone methods, the selected cells can be pre-picked for subsequent scanning. The control file lists for pre-picked and random co-ordinates are separate and maintained independently. Seek commands for co-ordinates in either list are also distinct.

Random co-ordinates are generated from a rectangular coverslip area whose position and dimensions are pre-definable by the user. This rectangular area is independent of any search zone in effect. For example, random co-ordinates may be generated from a large rectangular coverslip area measuring 10 X 10 millimeters and displaced 5 X 5 millimeters from reference mark 1. Then, by either random cell or random zone selection, cells may be sampled from the 10 X 10 millimeter area. The seek function where relative or absolute co-ordinates are supplied by the operator can be used to visually verify that this rectangle includes only usable coverslip area. Such verification would precede generation of random co-ordinates.

A technical problem encountered during the development of this part of the Scanning Subsystem is illustrated using Figure 6. Co-ordinates listed either as the result of pre-picking or random generation are not generally listed in an order which permits sequential passage in an efficient manner. This is particularly the case for random co-ordinates, which are initially listed in the order generated.

Figure 6 illustrates the problem for a small number of locations. In both Figures 6A and 6B, the point numbered 0 is the current stage location (position of the crosshair intersection). In Figure 6A the points numbered 1-5 represent 5 random locations in the order of generation and initial listing. Passage to these locations in the numbered ordering requires traversal of more than twice the distance as the order depicted in Figure 6B. Since the maximum stage speed is used in all seek operations, this means more than twice the traverse time is required just for positioning to the various locations. The cell scan rate using the order in Figure 6A would be less than that using the order in 6B because of the increased travel time. For cell samples numbering 50 or more, and over multiple samples, it has been discovered that one hour or more out of each 4 hour scanning session might be spent waiting on stage travel. For practical reasons relating to cell scan rate and anticipated statistical requirements, this overhead is considered unacceptable.

However a solution to the problem has been included in the Scanning Subsystem, a sorting function is provided which reorders the listed co-ordinates in relationship to the current stage position. The new ordering is such that the total distance traveled in passage through the points using list order and starting from the current position is minimal.

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In terms of Figure 6, though locations may be generated and initially listed in the order depicted in Figure 6A, they can be sorted and traversed in the order given in Figure 6B. Sorting may be performed on both random and pre-picked co-ordinates and is always relative to the current stage position (Point 0).

Randomly generated locations are automatically sorted, without operator request, immediately after they are initially recorded. Pre-picked co-ordinates are sorted only upon operator command. In the case of pre-picked co-ordinates, resorting is permitted with or without repositioning of the stage. When the pre-picked list is resorted after changing the current state position, the list order will generally permute to meet the minimal distance requirement.

Both the pre-picked and random co-ordinate lists can be printed to the operator's console at any time. An example of such printed listing is included as Table II. The table exhibits 40 randomly generated co-ordinates which have been (automatically) sorted. The generation took place with the stage positioned at the origin (0,0) using reference mark 1. The rectangular area from which the locations were generated is the box [-1,] X [-1,1]. The PAIR column specifies the list order in the control file. The XSTEPS and YSTEPS columns give the number of steps from the reference mark serving as the co-ordinate system origin. The sign designated for each number of steps indicates positive or negative direction along the respective axes. Though the physical stepping distance for the stage is 0.5 microns, it is possible to utilize any positive multiple of 0.5 microns for a logical search step distance. The logical search step distance used for the creation of Table II was 2.00 microns and is indicated in column STEPLEN. For the operators convenience, the equivalent millimeter co-ordinates are printed in column X-MMETERS and Y-MMETERS. The ORG column shows the number for the reference mark in current use. The listing is output in paginated format with at most 18 locations listed per page. The next page, if any, is printed upon the typing of a carriage return on the operator's keyboard. The right marginal space is intended for comments and annotation.

Through proper use of these listings, searching and selecting of cells can cross scanning session boundaries and be continued from operator to operator. It has already been mentioned that both arbitrary and sequential order can be followed in positioning to listed locations. In addition, sequential list order can be started at any position in the PIAR column. Thus, cell selection can continue in the next session where it was terminated during the current one. A running record for purposes of controlling the process from operator to operator is kept by annotating these printouts. Fresh printouts can be repeatedly made without affecting the list order or content.

The operator may specify a logical search step distance which is a multiple of 0.5 microns. Stage stepping during the SEARCH phase is in terms

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of integral numbers of these logical steps in both axes of motion. Co-ordinate data maintained in the SEARCH phase is kept internally as the number of logical steps from the reference mark in each axis. For purposes of operator printouts, this data is expressed in millimeters from the reference mark by multiplying the co-ordinate step numbers by the logical search step distance. When the operator commands a displacement in millimeters, the displacement data is converted to the nearest number of appropriate logical search steps. The physical stepping distance of 0.5 microns is largely transparent to the operator, since the programming automatically converts each required logical step into the necessary number of physical steps prior to movement.

The logical search step distance is independent of the interpixel distance used in generating digital cell images. Generally, the logical search step is larger, resulting in coarser motion and positioning. This is acceptable because the process of searching and selecting cells for scanning does not require the same spatial precision as the scanning itself. The advantage of coarser search steps is that greater areas may be covered using step counters implemented with 16 bit hardware arithmetic. For each co-ordinate axis, the limits of displacement from the reference mark in each axis is -16,383 and +16,383 steps in the respective axial directions. If logical search step distance were limited to 0.5 microns, then the limits of displacement millimeters would be approximately -8 and +8 millimeters in the respective axial directions. However, it is desirable to cover an entire slide from any of the four possible reference marks (See Figure 2). The logical search step approach provides this capability in the context of 15 bit integer storage magnitude characteristic of the minicomputer being used.

As a result of the extensive spatial coverage provided by this technique, approximation of the coverslip to any particular slide reference mark need not be particularly close. A given mark merely furnishes an origin regardless of the coverslip's distance from it. Sufficient stage range is available to move from mark to coverslip and, once there, traverse the coverslip's entire extent.

The location recorded for any selected cell is in terms of logical search steps, their designated length in microns, and the number of the reference mark used. For example, by recording (ORG, XCORD, YCORD, STEPLENGTH) = (2, -20, 100, 2.00) for mark number, X-co-ordinate, Y co-ordinate, and search step length, the cell location is complete. This is sufficient information to find the cell at any subsequent time. Such data is recorded on magnetic tape along with other parametric data items associated with the image.

When cell is selected and the eyepiece crosshairs placed upon it, an appropriate command records the location data. The operator may also cause the cell to be named. Naming can be performed in two ways. The operator may 1) type a 12 character identifier, or 2) cause one of up to ten previously stored, numerically indexed names to be used. In either case, the cell identifier is recorded with its parametric data.

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Numerically indexed names consist of 12 characters. The first eight can be letters or digits and the last four must be digits, 0-9. An example is DNPH32160201. The first eight characters are called the namestem and are used to specify the cell type in any convenient manner. In the example given the namestem is DNPH3216 and could be used to indicate that the test cell has been exposed to DNPH at 32 mg/liter for 16 hours. The last four numeric characters are called the index. In the above example the index is 0201. The index gives the number of the particular cell in the series with namestem DPNH3216.

Up to ten namestems may be predefined along with low and high limits for the index. When an operator chooses automatic naming, he specifies the namestem to be used. The next unused index value is automatically supplied and the entire name is recorded with parametric data for the cell. If there is only one predefined namestem, choice of automatic naming results in immediate synthesis of namestem and index into a single cell name with appropriate recording. The operator need not, in this case, supply even the namestem.

This facility allows 1) the identification of several differently named series during the processing of a single slide, 2) consistency in the designation of cell identifiers, and 3) non-redundancy in the assignment of the index portion. Past experience with cell identifiers recorded by an unaided operator indicates that misspellings and duplication of numerical indices are common--particularly for cells scanned towards the latter part of sessions where operator fatigue is an important factor. In this subsystem, the unaided method is retained, but the automatic device is provided to eliminate the errors mentioned.

The SEARCH phase is exited on operator command to the OPTION phase. From the OPTION phase, selection of the INITIALIZE, SEARCH, EDIT, or LOGOUT phases is permissible- when the previous phase was SEARCH. These selection constraints are placed in order to limit the operator's activities to those which are appropriate for the situation and, thereby, minimize the possibility of actions which result in errors of either commission or omission and nullify previous scanning work.

E. EDIT Phase

Once a cell is located and named, the SEARCH phase may be exited to OPTION and thence to the EDIT phase. The EDIT phase allows the interactive annotation of captured cytologic images through joystick and cursor type tracing, where the eyepiece crosshairs assume the role of cursor. This graphic editing is performed prior to execution of scan, and is, therefore, referred to as pre-editing. The advantages of this technique are discussed in a previous report (3).

Graphic editorial data is generated by tracing collections of plane curves. Each curve in the collection is one of 4 types (See Figure 7). Each plane curve is called a component and the four types are named PATH, EDGE, PERIMETER, and and POINT.

A PATH is a sequence of points in the image say $P(0),...,P(n)$. $P(0)$ is called the initium and $P(n)$ the terminus. Further, for any pair of consecutive points in the sequence, say $P(k)$ and $P(k+1)$, either $P(k)$ and $P(k+1)$ are distinct adjacent points or $P(k) = P(k+1)$. Two points are adjacent if $P(k) \neq P(k+1)$ and they are separated by precisely one interpixel step in any of the 8 possible discrete directions. Direction is indicated by link code, a number from 0-8. For further details on link codes see (4,8). When consecutive points are identical, say $P(k) = P(k+1)$, the geometry of the curve is unchanged by discarding $P(k+1)$. Therefore, it will be assumed hereafter that this has been done and $P(k) \neq P(k+1)$ for $k = 0,...,n-1$. Note, initium and terminus may be identical.

Curves which are PATHs can intersect themselves. That is, $P(j) = P(k)$ is permissible. Further, the definition for a PATH does not preclude that initium and terminus be identical, i.e., $P(0) = P(n)$, although in Figure 7-1 this happens not to be the case.

An EDGE is a PATH which does not intersect itself and is open ended. (See Figure 7-2). That is, if $P(0),...,P(n)$ is the sequence of EDGE points, then $P(j) \neq P(k)$ for all choices of $j \neq k$. This includes initium and terminus. See Figure 7-2.

A PERIMETER is a PATH for which initium and terminus are identical, but does not otherwise intersect itself. That is, if $P(0),...,P(n)$ is the sequence of PERIMETER points, then $P(j) \neq P(k)$ for all choices of $j \neq k$ where $j,k = 1,..., n-1$ and $P(0) = P(n)$. A PERIMETER is depicted in Figure 7-3.

A POINT is a PATH consisting of a sequence $P(0),...,P(n)$ where $n = 1$. The initium and terminus are, therefore, identical.

There is an hierarchy to which the four component types conform (Figure 8). Components of type EDGE, PERIMETER, and POINT are all PATHs. Further, POINTs are PERIMETERs. This allows a systematic computer checkout of curve properties whenever a component is generated and before it is accepted for recording with the image data.

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For practical purposes, only three kinds of checking are required. These are 1) for adjacency of consecutive points, 2) for intersection, and 3) for curve closure-i.e., terminus is identical to initium. These will be referred to as adjacency, intersection and closure checks, respectively. PATHs must pass the adjacency check. EDGEs must fail both the intersection and closure checks, but pass the adjacency check. A PERIMETER's interior sequence points, $P(1),...,P(n-1)$, must fail the intersection check and the entire curve must pass both the adjacency and closure checks. Points vacuously pass both adjacency and closure checks, but fail the intersection check.

Graphic editing constitutes tracing organized collections of components. Such a collection is called an edit set, or just set when understandable from context. An edit set is composed of edit groups, or just group for short. Each group consists of a collection of components. All the components in a group must be of the same type- i.e., PATH, EDGE, PERIMETER, or POINT. During the EDIT phase, multiple edit sets may be generated and recorded with the image. The collection of edit sets generated for the image is called the graphic data. This is to distinguish it from both the image itself and the parametric data.

Edit sets used during actual image editing are predefined. That is, the set and each of its groups are given names. The number of groups, maximum number of components per group and minimum number of components per group are also pre-determined in the edit set definition process. The component type characteristic of each group is fixed.

The operator traces group components, to a quantity numbering between the preset minimum and maximum, and in response to prompting by the EDIT phase program. The program sequences through a chain of requests for components based upon a subfile of edit sets which have been preselected for use with the cells being scanned.

The sets in a master edit set file are defined and placed there using other interactive functions in the EDIT phase program. Similarly, the selection function involves picking edit sets from the edit set master file for use with the cells being scanned and the programming for this function is also contained in the EDIT phase. Edit sets may also be removed from the master edit set file. To change the collection of edit sets selected requires only a repeat of the selection process with picking of the newly desired sets.

Additions and deletions in the master edit set file are not performed during scanning sessions even though the programming for these activities is contained in the EDIT phase of the Scanning Subsystem. Generally, project supervisory personnel use this aspect of the subsystem prior to scanning. In this manner, instructions concerning the amount and type of editing to be performed by the operator are built into the subsystem by the time the operators begin to scan. All selected sets in the graphic data for the cells in question must be completed. Attempts to scan a cell in the presence of incomplete graphic editorial data will be rejected.

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The edit set and its group names are used in construction of message prompts to the operator for component tracing. For example, there is an edit set named CELLNUCLEI. It consists of one group named NUCLEAR. The NUCLEAR group is defined to contain a minimum of 1 and a maximum of 100 perimeter type components to handle single and multinucleation. The operator is prompted by printing to the console:

CELLNUCLEI: TRACE NUCLEAR PERIMETER n

where CELLNUCLEI and NUCLEAR are taken from the predefined edit set and group names and n is the integer sequence number of the perimeter being traced. This number must be between the minimum and maximum defined for the group- from 1 to 100 in this example. The word PERIMETER in the prompt is derived from a code for the component type of the group. This code is recorded as part of its definition. Similarly, for an edit set named CELLNUCLEOLI, with one group named NUCLEOLAR and consisting of components of POINT type, the prompt would be:

CELLNUCLEOLI: TRACE NUCLEOLAR POINT n

where CELLNUCLEOLI, NUCLEOLAR, POINT, and n are obtained in a manner analogous to that in the previous example. In this case, the minimum number of components is 0 and the maximum 10. This covers the cases of multinucleolation and non-visible nucleolus.

All edit sets defined in the master edit set file have a unique positive integer designation in addition to a 12 (or less) character name. All groups within a set are similarly sequenced with integers. The sequence of operator prompting threads through the selected edit set number until all selected sets are completed. Within each set, prompting sequences through the groups in order of increasing group number. In each group, prompting continues until either the maximum number of components are obtained or the operator signals the group is completed, based on visual assessment of the cell. Components are numbered in the order traced. For example, a cell with two nuclei has the NUCLEAR group completed after two nuclear perimeters.

In no case may an operator terminate a group before the minimum number of group components has been traced. Any attempt to do so results in rejection of termination and re-prompting for another group component. Once a group is validly completed, the prompting proceeds to the next group in sequence for the set. When the set is complete, the prompting proceeds to the next set in the selected graphic data. When the graphic data is complete, the operator is informed that editing is complete.

Assume, for example, that two sets have been selected from the master edit file for use in editing the cells being scanned. Let one set be CELLNUCLEI, which has the set number 30, and the other be CELLNUCLEOLI, which has the set number 50. These sets are defined as previously described. Operator

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prompting begins with the smallest set number among those sets selected as part of the graphic data. It proceeds in order of increasing set number, but these numerical designations are invisible to the operator. The operator sees only the names for sets, groups and components. The first prompt will be for set 30, group 1. Since group 1 has from 1 to 100 components of the PERIMETER type, there will be from 1 to 100 prompts in completing this set depending upon where the operator terminates. A minimum of 1 perimeter must be traced before termination of the group will be allowed.

After set 30, CELLNUCLEI, is completed, prompting for set 50 begins at group 1, which is also the only group in the set. This group contains from 0 to 10 point type components. When the group is validly terminated the set is complete and so is the graphic data.

The sequence of prompts and operator responses is given below. The assumptions are that a cell with 1 nucleus and 3 nucleoli has been selected. The dialogue between operator and scanning subsystem is:

CELLNUCLEI: TRACE NUCLEAR PERIMETER 1
Operator traces nuclear perimeter.

CELLNUCLEI: TRACE NUCLEAR PERIMETER 2
There is none, so operator terminates the group. This completes the set, and prompting proceeds to the next one.

CELLNUCLEOLI: TRACE NUCLEOLAR POINT 1
Operator traces a nucleolar point (puts crosshair intersection on it). But there are more, so the prompting for the group continues.

CELLNUCLEOLI: TRACE NUCLEOLAR POINT 2
Same as previous operator response.

CELLNUCLEOLI: TRACE NUCLEOLAR POINT 3
Same as previous operator response.

CELLNUCLEOLI: TRACE NUCLEOLAR POINT 4
There is none, so operator terminates the group. This completes the group, set, and graphic data and the subsystem responds appropriately.

END OF GRAPHIC DATA FOR DNPH32160217

The last message in this scenario indicates that the name of the selected cell is DNPH32160217, given during the SEARCH phase as described previously.

Because of the way in which edit set, group, and component names are used in the prompting process, they should be chosen so that the abstract form:

setname: TRACE groupname cpntname n

is as close to natural language sentential form as possible. Here setname

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and groupname are character strings from 1 to 12 in length, designating set and group respectively. The element cpntname will always be PATH, EDGE, PERIMETER, or POINT. The integer n is the component sequence number within its group and lies between 0 and the maximum number permissible. Generally, setname is chosen to be a stand-alone or titling type of name (e.g., CELLNUCLEI). The identifier for groupname may be chosen in a manner consistent with a grammatical pronunciation. For example, the group name NUCLEAR in the set CELLNUCLEI is chosen rather than, say NUCLEUS.

The adjacency check, which is applicable to all component types, is performed in real time as the operator traces. When applicable to the component type, intersection and closure checks are performed at the conclusion of tracing. The requirements for passage or failure of the check depend upon what type of component tracing has been prompted. If the actual curve tracked does not satisfy the checks, the tracing is rejected and the operator informed to repeat it.

Since only four basic components are used, operator familiarity is quickly developed. Further confining tracing to these four graphic structures allows for systematic and greatly simplified error checking. It is possible to build edit sets from groups of these four basic components and, thereby, organize the graphic pre-editing data into a simply structured format. This format plays a major role in subsequent analysis of the cell images and in the construction of production systems.

The basic components produced during tracing are recorded using a chain code data structure. This technique is widely used in image processing and is particularly suitable for the acquisition, recording, and processing of line drawing type data. A survey of this and other techniques is given in (8). Essentially, each traced component is recorded by specifying the co-ordinates of the initium, followed by link direction codes (0-8) which indicate the displacement needed to get from one point to the next adjacent one in the component's point sequence, $P(0),...,P(n)$.

Since the linear trajectories involved are obtained from tracings on natural images (i.e., cells), long runs of identical links are infrequent. Therefore, all links are recorded explicitly and "run length encoding" schemes are not utilized. Long vectors forming straight lines between two points are kept in the form of separate point components indicating start and end of vector. The amount of memory required for the storage of each link code is 4 bits. The link codes 0-8 indicate direction (8 indicates no displacement) and the numbers 9-16 are used to encode signal data used in processing components.

In spite of the small number of allowable components, a wide range of cell image annotation is possible. Nuclear and cytoplasmic borders can be outlined and nucleolar pointers placed. When multinucleation and multinucleolation is visible, multiple perimeters and pointers may be placed. More complex edit sets consisting of several groups, each with different characteristic components can be defined and used to annotate more complex cytostructural detail.

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Special mention should be made with respect to the generation of perimeter type components. Perimeters are essentially curves which intersect only at initium and terminus. Since the performance of such tracing is done by observation through the microscope and no outline of points already traced through is visible, the operator cannot be expected to stop tracing at exactly the point it was begun. That is, the unaided operator is unlikely to cause initium and terminus to be identical.

To compensate, there is an algorithm included in the closure checking software which first closes an open trajectory-if the subsystem requires the current component being traced to be one. This closure operation is done in such a way that the area interior to the completed component is maximally incremented by a right angle corner added to the trajectory which closes the remaining gap. This strategy is adopted to reduce the possibility of cutting of part of the structural details which the perimeter is supposed to encircle. It is possible for this algorithm to close the hitherto open curve in such a manner that the overall curve intersects itself at other than initium and terminus. This would render the curve unacceptable as a perimeter.. If this occurs during <u>forced</u> closure, the intersection check will detect it and reject the curve.

The operator reduces the likelihood of such rejection by starting and ending perimeter tracing with reference to some observable structural detail. In such a manner it is possible to approximate the starting point fairly closely when terminating a perimeter. The likelihood of forced closure generating an unacceptable perimeter is reduced by such approximation.

At least one perimeter must be generated for every cell scanned. This perimeter belongs to an edit set numbered 1 and named SCANPERIMETR. The set contains one group numbered 1 for the edit set and named SCANNING. This completes the contents of the set. The operator prompt given by the subsystem is, therefore:

SCANPERIMETR: TRACE SCANNING PERIMETER 1.

The minimum and maximum number of components for the SCANNING group is 1. Therefore, the operator must trace precisely one scan perimeter per cell. The scan perimeter edit set (number 1) must be selected as an edit set in the graphic data.

The scanning perimeter should be traced in such a manner as to exclude from its interior all objects in the microscope field other than the cell of interest (See Figure 9). This is the major mechanism by which unwanted neighboring detail is edited from the computer image obtained during scanning.

All graphic data generated during the EDIT phase is recorded with respect to the co-ordinate system determined by the cell location as origin and funda- mental step length equal to the <u>interpixel distance</u>. This distance must be a multiple of 0.5 microns and may be specified as a parametric data item enterable during the EDIT phase. The interpixel distance is not necessarily identical to the logical search step length used during the SEARCH phase.

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Because the scale used for the digital image and graphic data is the same (i.e., one step length equals the interpixel distance), the graphic data may be viewed as an annotative overlay of the image. Every recorded component for each edit set included is positioned in the image relative to the cell location which is used as the origin of scan. The origin-of-scan and the cell location are the same absolute position on the slide. However, the cell location is in terms of logical search step co-ordinates and the origin-of-scan is the same absolute position used as the center of a virtual co-ordinate system with the interpixel distance as the fundamental distance between points on the X and Y axis.

Other edit sets which are generally used, but nonetheless optional, are CELLBORDER (set number 10) and CELLNUCLEI (set number 30). For many experimental runs, SCANPERIMETR, CELLBORDER, CELLNUCLEI, AND CELLNUCLEOLI will be sufficient graphic editing data and together constitute the graphic data. Other requirements can be met using the edit set defining capability and the set selection function, both of which are included in the EDIT phase software as previously described.

The EDIT phase is exited to the OPTION phase. When having previously exited the EDIT phase, OPTION permits the selection of the INITIALIZE, SEARCH, SCAN, or LOGOUT phase. The restricted selection is again provided to minimize operator error by limiting choices to those which are conceivably appropriate for the situation. Note, for example, then the option to scan is not provided except after editing. A cell must have, at least, a scan perimeter provided before scanning is possible.

F. The SCAN Phase

The SCAN phase allows the operator to accomplish: 1) the entry of additional parametric data, 2) the performance of the actual physical scanning of the cell, and 3) the output of cell image along with associated parametric and graphic data to magnetic tape. The entry of additional parametric data is performed interactively on a request-response basis. Physical scanning, after being commanded by the operator, is performed automatically - without interaction between subsystem and operator. Data output to magnetic tape is performed upon command from the operator and must be preceded by a successful physical scan.

The SCAN phase software is designed, but not coded at this writing. Therefore, only a brief description of intended operation will be given in this document.

A SCAN phase subdirectory for parametric data entry controls the particular items to be entered. The operator must fill all required items, but not all items need be filled each time a cell is scanned. The operator may alter any data item controlled in the SCAN phase parametric data subdirectory. Attempts to scan with unfilled parametric data entries are rejected. In general, the type of parametric data entered during SCAN phase is that obtained at the point of actual scan and which requires update based upon microscopic observations in the immediate locale of the cell.

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The exact nature and amount of this data is determined by use of the Data Directory Editor and Data Subdirectory Creator programs in Category 4 of software support (See Table 1). As for parametric data entry in other phases of the scanning subsystem, it can be varied without additional programming through use of the software mentioned.

The first action taken by the subsystem after a command to scan is a check for the completion of all required parametric and graphic data. This check is global. That is, parametric data must be complete regardless of the phase in which it is entered. All other phases perform a similar check on parametric data, but only for that data which is enterable during the particular phase. Similarly, graphic data must be complete-every selected edit set must have its components traced. Any existing data gaps result in a rejection of the scan attempt with appropriate operator's message. In this case the operator must exit the SCAN phase and return to the phase(s) required to complete the image associated data.

If all associated data is complete, the cell is scanned. First, a scan pattern is established. This is done by utilizing the scanning perimeter edit set which was completed during the EDIT phase (edit set SCANPERIMETR). From the scanning perimeter, a framing rectangle is determined (See Figure 9). The axial limits of the framing rectangle, XMIN, XMAX, YMIN, and YMAX, are obtained from the analogous limits of the scanning perimeter. All co-ordinates and distances are relative to the cell location as determined and recorded during the SEARCH phase. Steps relative to this location, also referred to as the origin of scan, are in terms of interpixel distance. The inter-pixel distance is a parameter which may be entered as parametric data during the EDIT phase. Once entered, the interpixel distance needs be changed only when required. It need not be specified for every cell.

Once the framing rectangle dimensions are determined, an image data file is preconditioned to accept the digital picture. From the framing rectangle dimensions and the interpixel distance, the number of pixels per scan line and the number of scan lines are calculated. The scanning perimeter, as all graphic components, is traced to a scale in which all distances are multiples of the interpixel distance- not the logical search step distance. Therefore, the results of arithmetic done to calculate number of pixels per scan line and number of scan lines is precise. No roundoff or truncation error is involved. Based on this number of pixels per line and number of scan lines, appropriate storage in the image data file is allocated and all pixel values are set to zero.

After the image data file is preconditioned, the positions of all pixels within the framing rectangle which are on or interior to the scanning perimeter are calculated. These positions are recorded in such a way that, for each scan line crossing the framing rectangle (See Figure 9), those pixels on or interior to the scan perimeter can be distinguished from those which are not.

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Having recorded for each scan line the pixels on or interior to the scanning perimeter, a step-and-measure meander pattern is executed so that only the pixels on or interior to the perimeter are evaluated. As these pixel values are obtained from the microdensitometer, they are recorded in the image data file, replacing the previous zero value in the appropriate position. The result is that all pixels on or interior to the perimeter have measured values which are non-negative. All pixels within the framing rectangle exterior to the perimeter have zero value. Since the scanning perimeter lies outside the cell border, there is a rim of slide backround recorded. This finished picture is rectangular and free of unwanted neighboring detail, since the scan perimeter was traced to exclude these details from the scene digitized.

During the scan, a meander pattern is used to reduce the scan time by passing only once over each pixel requiring measurement. Analogous to the meander pattern used in the SEARCH phase, the major axis of meander is automatically alternated to more evenly distribute wear to the X and Y motors of the stage. This means that every other image is rotated by 90° in orientation with respect to its predecessor and successor. Since the cell image information sought is invariant under rotation, this does not compromise the subsequent analysis.

Due to the meander pattern of scan, every other scan line's data is returned from the microdensitometer in reversed order of pixels. The SCAN phase software automatically records the line in the right-reading order and not the order of scan. This is performed without additional disc processing, since each scan line is input to computer main memory prior to recording in the disc image data file. Scan data for a single line is input in one line pass to a main memory data buffer; then, depending upon the direction of scan, the data is output in the appropriate order to the image data file.

An additional advantage of buffering the scan line input, one line at a time, is that main memory requirements for holding image data during scanning can be kept small, leaving more room for program by requiring less for data. The traditional space/time trade-off is made, in this instance, favoring memory space saving at the expense of speed. However, given the relatively slow rate of scan due to the mechanical movement, the additional slow-down is comparatively negligible. On the other hand, the greater amount of space for program instructions allows a SCAN phase program which can perform the operations described in a relatively modest sized minicomputer's memory. The operations, inclusive of riddance of unwanted detail, meander pattern, and alternation of major axis to distribute wear evenly are far more valuable assets than the small amount of speed which would be gained by a higher degree of input image buffering. The pre-editing alone obviates need for post-editing, which can require an entire subsystem to perform-not to mention the operator time. Further, such image cleaning is performed on a displayed facsimile of the computer image instead of, as in this system, on the real scene being digitized.

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Once the scanning of a cell has been successfully completed, as indicated by a message, the operator may command that all the image data be output to magnetic tape. This includes the parametric, graphic, and image data. If output to tape is commanded, the format is one which has been designed for the project and adopted as a system-wide convention for storage of image and associated data on magnetic tape. This format is described in a preliminary definition document which has been forwarded to USAMRDC (2).

The SCAN phase is exited to the OPTION phase. When the previous phase to execute was SCAN, OPTION allows selection of INITZE, SEARCH, EDIT, EXTRACT, or LOGOUT. Entrance to the EXTRACT phase is only permitted after SCAN. SEARCH is re-entered when the next cell for scanning must be selected (or moved to, if preselected). A cell may be re-edited and scanned by returning to the EDIT phase. Changing various initialization settings is possible by a return to INITIALIZE and termination of the scanning session requires the selection of LOGOUT.

G. The EXTRACT Phase

The EXTRACT phase may be entered from the OPTION phase only after the successful completion of a cell scanning. This phase allows the extraction of specific cellular features- as identified by the operator in appropriate commands. The features chosen for extraction must reside, in the form of subroutines, in the system feature library.

The twofold purpose of the EXTRACT phase is 1) to permit the development of feature extraction software, and 2) to allow exploratory evaluation of specific features.

This phase is present in the Scanning Subsystem only as a program stub at this writing. Its further development will parallel the implementation of feature extraction and analytic software during the analytic phase of the project.

Exit from the EXTRACT phase is to OPTION. Following the EXTRACT phase, OPTION permits selection of the INITIALIZE, SEARCH, EDIT, or LOGOUT phases. It is re-emphasized that EXTRACT is enterable only after a cell has been successfully scanned.

H. The LOGOUT Phase

The LOGOUT phase is entered when it is desired to finish a scanning session. The scanning session consists of all activities performed from the entry to LOGIN phase and ending with the completion of the LOGOUT phase. Prior to powering down the subsystem, the LOGIN phase must be executed.

When the LOGOUT phase is executed, all parametric data which must change from session to session is erased from the files. Examples are: scan date, operator's identification, slide number, reference mark number, and scanning hardware switch settings.

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The scanning control file items are set to appropriate conditions, indicating the system has been powered down and there is a need for re-initialization upon start-up. Re-initialization involves such things as: slide alignment, reference mark identification, pixel integration switch settings, magnetic tape loading and positioning.

The LOGOUT phase also assigns the unit numbers for system peripherals to the system console for all units except the operator's scanning console. This having been completed, LOGOUT returns to the OPTION phase. The LOGOUT phase runs completely automatically. No operator interaction is provided.

When OPTION phase executes after a LOGOUT phase is complete, only two choices are made available to the operator. The first is to enter LOGIN again; the second is to STOP the system with an end-of-job. The only Scanning Subsystem phase enterable from OPTION after the LOGOUT phase is LOGIN.

When the command to STOP is given in the OPTION phase after logging-out, the subsystem prints a termination message to the operator's console. Prior to ending the job (i.e., scanning session), the operator's console units are re-assigned to the system console (a separate teletypewriter). Then the end-of-job is executed. The entire minicomputer system is, at this point, insensitive to typing on the scanning console. It will respond only to commands given from the system console- from which non-supervisory personnel are restricted.

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SYSTEM DESIGN AND ITS RELATION TO THE DEVELOPMENT OF A CYTOTOXICOLOGIC WATER ASSAY

This system is intended as a research resource for the obtaining of data required in implementing production systems for the detection of noxious chemical entities in water. The underlying hypothesis is that test cells, when exposed in culture to water containing noxious agents, undergo morphologic alterations. It is further hypothesized that computer images of cells which are so exposed reflect such changes, and that the cellular morphologic responses are graded with respect to both duration of exposure and concentration of noxious agent(s). The feasibility and sensitivity of cytoassay for water toxicity based upon this hypothesis can be tested using data obtained using an appropriately designed, light microscopic, computer image acquisition and analysis system.

A system designed to investigate this hypothesis must have certain general characteristics. It must enable the acquisition of light microscopic, computerized cellular images as well as associated experimental data not obtainable from computations performed on the digital image itself. It must be sufficiently flexible to allow usage under various experimental conditions. Computer image analysis must not depend upon a prior knowledge of appropriate techniques for the preparation of cells for microscopy. This is particularly important with respect to fixation and staining technology.

Such a system should yield quantitative results, since subsequent automation of a cytoassay depends heavily on quantitation. The morphologic alterations hypothesized should, therefore, be amenable to numerical description. Such numerical morphologic descriptors are called features, and the system should permit an efficient search for a workable set of features indicating cell intoxication.

Principal elements among the efficiency requirements are: 1) the system should allow the exploration of features for which there is prior evidence suggesting sensitivity to noxious agents, and 2) the numerical evaluation of the features should not depend upon the automatic location in the computer image of cellular infrastructure used in the computation. Prior evidence of feature sensitivity can be obtained from qualitative light and electron microscopic investigations on cells exposed to model toxicants. Independence of automatic cytostructure location is required, since such automation is dependent upon densitometric patterns in the image. Such patterns are, in turn, dependent upon specimen preparatory techniques, spatial quantitization (resolution) used in digitizing the cellular image, and upon densitometric quantitization (resolution) used in expressing pixel values.

A larger number of features will probably have to be tested for sensitivity as cytotoxic indicators than will ultimately prove useful for the purpose. It is more expedient to invest an effort in automatic location of only those cytostructural elements which are required in the computation of useful features. Further, because the promotion of feature sensitivity and automatic location may have competitive influences on staining and fixation

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methods, a strategy which gives priority to finding features and preparatory methodology which give good cytotoxic discrimination is more propitious. Automatic location algorithms can then be devised, which have as their target the finding of a limited set of structures known to be needed in feature computation.

The conditions of satisfactory feature discrimination among intoxicated cells are those under which automatic structure finding should be carried out- not the reverse. An approach which first optimizes conditions for automatic structure location may result in effort wasted in finding either unnecessary structure or structures which yield non-discriminatory feature values under preparatory techniques which favor location.

A research system for this type of investigation should also enable acquisition of sufficient numbers of cell images to meet the statistical requirements for dose and time response testing of cellular morphology as indicated by feature extraction. However, this image data rate need not be of the same magnitude as that required in a production environment for a workable cytoassay. Certainly, data precision should not be sacrificed to speed- as long as sufficient numbers of cell images for experimental purposes can be obtained within practical laboratory time constraints.

In addition to a workable set of cytomorphologic features, significant parameters governing preparatory and scanning procedures must be identified and evaluated. During research and development, these parameters must be faithfully and consistently recorded in association with the images to which they apply. Such associated parametric data reflect culture, fixation, staining, slide mounting, optical, and electronic conditions under which each image is obtained. In the face of fairly numerous computerized cell images, it is important to insure these data do not become separated from the image to which they apply.

Unlike cytopathologic technique applied for other purposes, such as screening for disease in the female reproductive tract, formal sample-theoretic considerations play a more prominent role in cytoassay. In the case of pap smear screening, the entire cytologic smear is evaluated by eye. If the number of cells present in the smear is sufficient and some of them indicate a specific cytodiagnosis, e.g., carcinoma-in-situ, then such a report is justified. In routine clinical applications, the smear is not used in making quantitative inferences about the parent tissue cell population. Nor is such quantitation necessary for diagnosis, since a little cancer is enough.

In the case of cytoassay, however, a sample of cells cultured under specific conditions is used to make statistical inferences concerning quantitative morphologic trends in the parent population. It is anticipated that the relative toxicity of a water sample can be gaged in terms of the intensity of cellular feature alterations and the frequency with which these

alterations are found in the population. This requires random sampling of cells for digitization. Whereas, in pap smear screening, it is permissible for the cytologist to become attracted to and base judgment upon specifically those cells manifesting atypia suggestive of disease, the selection of cyto-assay cells which catch the eye would lead to a biased sample and invalid inference.

Therefore, a system for cytoassay development should promote the required unbiased sampling. This is particularly important in two aspects: 1) cell selection should not be a function of attractiveness to the human eye, and 2) cell selection should not be confined to particular areas of the microscope slide. Another consideration, solved essentially by the culture technique, is that manipulative methods used to obtain cell populations and prepare them for microscopy should not differentially destroy or lose intoxicated cells. Facilities assuring 1) and 2) above should be available when each microscope slide is searched and cells are selected for scanning.

The system constructed for this project takes the notions discussed above into consideration. Prior to reading the remainder of this section, the contents of the sections entitled SYSTEM HARDWARE, SYSTEM SOFTWARE, and SCANNING SUBSYSTEM OPERATIONS should be familiar to the reader. The remainder of this section is devoted to matching specific aspects of the system with the requirements discussed above.

The performance of scanning microdensitometry using a mechanical stepping stage for slide movement (Figure 1) is much slower than flying spot and video methods. However, the photo-optical equipment used for the scanning hardware was previously available and, in the interests of keeping system construction costs down, this technology has been selected.

In order to achieve cell digitization rates of 0.5 to 1.5 minutes per cell (Mouse-L) an improved stepping stage has been acquired which, in combination with a meander pattern of scan (Figure 5), will reduce physical scanning time to one-half that required for comb scan of the same raster. The computer can re-order every reversed line, rendering the image lines right reading, in a negligible amount of time compared to that taken for stage return in comb scan patterns. The result is that each pixel is traversed only once and, due to this factor alone, the scanning time is half that of a comb scan.

The scanning perimeter technique, which serves an important editing function, also increases speed of scan. Though the final computer image of each cell is a rectangular array of pixels which is co-terminal with the dimensions of the framing rectangle (Figure 9), only the scan line segments interior and on the scanning perimeter are traversed during meander. This removes from 10 to 25 percent of the raster's pixels (Figure 9) from the traverse and further reduces scan time. The scan is faster because there is less to scan. At the same time, unwanted neighboring detail is eliminated from the picture.

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The motorized stage's stepping speed of 200 steps per second together with the use of small, 8mm X 22mm, coverslips allows traverse of the longest coverslip dimension (the diagonal) in less than 3 minutes. This is particularly important when co-ordinates for cell selection are either pre-picked or randomly generated.

Seek times from location to location are further minimized by taking the path of least traverse time between any two points. When seeking to each location in either a pre-picked or randomly generated list is required, the order of traverse may be permuted to obtain a much reduced total travel distance- and, therefore, reduced total travel time. The overall result is that the time spent seeking from cell to cell is brought to within practical limits, even though the cells being scanned are distributed uniformly over the entire coverslip.

Ordering location lists to reduce total travel time, traversing paths between locations which are the shortest, meander scanning patterns, and limiting pixel measurement to only those lying within the scanning perimeter are direct approaches to increasing overall scanning throughput. A major indirect approach for increasing throughput is embodied in the use of the scanning perimeter concept. The need for a separate data processing stage to clean up digital images is eliminated entirely.

Due to the presence of near-lying cells and debris, it is frequently not possible to frame a selected cell in a rectangle which excludes the unwanted objects. Such unwanted detail interferes with subsequent analysis and so must be removed from the picture or ignored. Systems which are constrained to scan a full rectangular raster, regardless of their speed, frequently require a follow-up editing procedure to rid the picture of included, but unwanted detail. The process is further slowed if the clean-up is performed on an image facsimile (e.g., a CRT display) using some sort of interactive graphic device (e.g., a light pen). Cumbersome comparisons between a photomicrograph taken at scan time and the displayed image facsimile, which may not be particularly good, have been used to identify portions of the unedited image which contain extraneous detail. The process can be slow and tedious-not to mention any time lag involved in awaiting computer time in situations where a dedicated machine is not used for the task and timesharing is either unavailable or ineffective. Further, if the facsimile is poor, there is a danger that uncertain identification of the selected cell's outline will result in erroneous editing and compromise the value of the image. Thus, a very fast scanning system which requires this type of image processing can lose most or all of its speed advantage in a slow post-editing process which may compromise the image data.

In this system, pre-editing of the cellular image is performed through use of the scanning perimeter and other edit sets available during the EDIT phase of scanning. To be sure, additional time is required to perform this task, but setting up another entire image processing stage, working with image facsimiles,

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and waits for machine time are eliminated. Since the scene being edited is the original, and few substitute presentations of the cell can match its appearance through a research quality microscope with good illumination and highly corrected optics, faster and more accurate editing is possible.

Systems which locate and isolate cells completely automatically do not require additional post-editing, but among examples known to the authors, are dependent upon pre-determined specimen properties. These properties may involve the nature of the cell sample (e.g., a peripheral blood monolayer), the staining technique used (e.g. Feulgen reaction with special cytoplasmic counterstain for contrast), or both. For full automation, such systems require some form of automatic focus- especially high resolution for scanning.

Because digital images can be examined by the extraction of numerical morphological descriptors (features), the system is capable of yielding quantitative results which might be used in the implementation of future production versions for the performance of fully automated cytoassay. Since all data capture is on-line and in real time, these data, whether parametric, graphic, or the image itself, are checked before acceptance and recording. When invalid pixel data is received from the microdensitometer, it is automatically rejected and the measurement is repeated. If the operator supplies invalid parametric or graphic data, an error message to that effect is printed on the scanning console and the operator must repeat the entry. Thus, the system promotes the acquisition of hard data, both automatically and inter-actively. These provisions for data integrity are especially important considering the relatively large volume of data collected. Retrospective data fixes become increasingly impractical as data volume increases.

The totality of data collected is conceptually divided into two classes: 1) image data (the digital images), and 2) image associated data. Image associated data is further classified as either parametric or graphic. At the finest level of data classification, therefore, a particular datum is either image, parametric, or graphic in type.

The principal function of parametric data collection is to maintain in association with each image a specification of pertinent image attributes which are not conveniently, or even possibly, obtainable from the image itself. Examples are: operator's identification, scan date, slide identification, fixative, stain, cell identifier, interpixel distance, cell location, reference mark number, scanning magnification, scanning numerical apperture, time-in-culture, exposure concentration, model toxicants (if any), pixel integration factor (every pixel measurement may be replicated with the average returned from the microdensitometer), bandwidth of illumination, number of pixels per line, and number of lines in the digital image. This list, which is by no means exhaustive, contains parametric data items referring to a wide range of experimental conditions. Also included is information required by the system to access the data in the format recorded on magnetic tape and disc- e.g., number of pixels per line and lines per image. Lastly, system bookkeeping information,

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though overhead, is required for supervisory control of data acquisition. For various reasons it becomes necessary to know who scanned a particular cell(s) and on what date. Hence, this and other data relating to hardware switch settings, though not used in the cell image analysis, are maintained for every image.

Parametric data maintenance is particularly critical in a research and development situation, since there are numerous items to be kept whose values cannot be presumed fixed- as they might be in a stable production environment. Because of the volume and variation, three potential problems must be dealt with: 1) data validity, 2) consistent and comprehensive recording in a uniform format, and 3) insuring that numerous parametric items be recorded with each image, but not require prohibitive amounts of time in doing so.

Data validity provisions have been previously discussed in terms of on-line, real time checking. Comprehensive, consistent recording and reduction of input time have also been addressed in the design and implementation of this system.

Parametric data is banked in intermediate disc files for output to magnetic tape along with the digital image of each cell. All of this data must be entered once, but most of it does not change from cell to cell or from session to session. After the successful completion of a particular cell scan, and on the operator's command to record the image, the image and all associated data are recorded on magnetic tape. The only option available to the operator is to record or not. There is no requirement for remembering to record image and associated data separately. Since all required data items must be filled for the record command to be obeyed, consistency and comprehensiveness is forced by the system. The recording format is uniform, since it is controlled by program.

Parametric data entry time is greatly reduced, since only a small number of items need be altered at appropriate times by the operator. Wherever the system can anticipate the need for such alteration, it requires the operator to do so. The parametric data is entered in a phase oriented fashion. That is, each scanning phase has a subset of parametric data items associated with it. During a particular phase, the operator can manipulate only those items associated with the phase. Any unfilled data item associated with the phase must be completed before it is exited.

Generally, phase association of parametric data items is done in a manner which tends to prevent certain errors. For example, interpixel distance is entered or altered only in the EDIT phase. This parameter is critical during both the EDIT and SCAN phases- as it is for subsequent analysis of the image. Its value must be the same for both editing and scanning operations, since the edit set group components must have the same spatial resolution as the image. To assure this, the interpixel distance is not alterable during the SCAN phase and the SCAN phase can only be entered after the EDIT. Thus, to scan one must edit and both must be done to the same spatial resolution. The operator is not given an opportunity to either scan unedited cells or edit

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and scan at different interpixel distances. This sort of control contributes to data validity, comprehensiveness, and consistency.

The need for data alteration is anticipated by building into the programming certain assumptions concerning what has transpired during each scanning phase. For example, if the operator has given a command to record the image and associated data on magnetic tape and that operation has been successfully completed, the parametric data item for cell name and location is flagged as empty. When the next cell is selected during the SEARCH phase, a new name and location must be recorded. This operation can only be performed during SEARCH and, since all parametric data associated with a phase must be complete before exit is allowed, the operator will be forced to supply these values. Similarly, execution of LOGOUT phase at the completion of a scanning session causes emptying of such items as operator's identification and scanning date. Entry of new values for these items will be forced during the next LOGIN phase. The two phases which have most to do with automatic emptying of data item values in anticipation of the need for change are SCAN and LOGOUT.

The system is not foolproof, however. It is possible to enter formally valid data which is incorrect. For example, the system has no way of determining that a slide identifier, say HEHASBLOWNIT, is incorrect. The system expects a 12 or less character, alphanumeric string for this item and the above example satisfies the criteria. The same applies to other parametric data items. An opportunity to detect such data aberrations is provided in all phases through a data listing facility. Using it, the operator may list the data currently recorded for the phase on the scanning console. Any errors can be fixed using various editorial commands provided. The commands include those for fixing individual errors and those for sequencing through all items associated with the phase. The latter mode of parametric data entry is particularly useful for initial entry, when all items are considered empty or incorrect.

In recognition of varying requirements for parametric and graphic data due to the experimental nature of the application, both the parametric and graphic portions of the data base are expandable. For paramatic data, such augmentation is performed using the DATA DIRECTORY EDITOR and DATA SUBDIRECTORY CREATOR programs in Category 4, Table I. For graphic data, edit sets may be defined and selected for particular scanning runs using facilities programmed into the EDIT phase.

All statements concerning system provisions for the comprehensiveness, consistency, and validity of parametric data apply analogously to graphic data. Graphic data is also checked on-line and in real time. All such data must be generated during the EDIT phase before exit is allowed.

A significant difference exits in the mechanism provided for display of graphic and parametric data. Parametric data is listed in printed form. Graphic data is displayed by playback. Any edit set group component, either at the time it is traced or after acceptance and recording, can be displayed by

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a command to drive the stage through the trajectory which has been previously traced. The operator can observe the trajectory as indicated by the motion of the eyepiece crosshairs relative to the specimen. This method 1) allows observation of the tracing in the image to be digitized, 2) eliminates the need for special graphics hardware (e.g., a graphics CRT), and 3) does not require hardcopy (generates no paper).

The interactive graphic editing provisions impact its flexibility as a tool for assessing both the feasibility of a cytoassay and the development of various data which would be required for implementation of production systems. Much of this has to do with the facts that 1) previous knowledge of highly discriminating features is non-existent and 2) standards for specimen preparation are not yet determined.

The system includes a means of recording graphic editorial data constructed from 4 simple geometric curves which are called components. Graphic data can be so recorded regardless of how it is generated. A means to generate such data interactively is provided for independence of "machine compatibility" specimen criteria. Such criteria must, generally, be satisfied to enable full automation - particularly with respect to the finding of required cellular structures. Whether obtained automatically or interactively, the organization and type of graphic data will impact in a significant way: 1) subsequent analysis of the image, 2) the extent of data checking required, 3) the logic complexity used in software or hardware programs for this or any production system, 4) cost, and 5) reliability. Some of these factors will be further discussed.

Analytic routines which have access to this graphic data can put it to a wide variety of computational uses. Areas within perimeters are measurable; lengths of paths, edges, and perimeters can be computed; distances between points can be calculated. Total optical mass of regions interior to perimeters is measurable by summing densities associated with interior pixels.

Density gradients, a knowledge of which is required for the development of automatic boundary finding software and hardware, can be calculated across edges and perimeters. Slightly more complex geometric calculations between heterogeneous component groups can yield measures of cellular features usually assessed only in a qualitative manner. For example, given a nuclear perimeter, it is possible to compute its geometric center. The distance from the geometric center to each point on the perimeter is then determined. The absolute value of the difference between the smallest distance and each of the previously calculated distances are summed. For a near round nucleus this sum will approximate zero. Nuclei with significantly off-round configurations yield positive summation values.

There are numerous ways that roundness can be measured; this example is but one- and not necessarily the best for the application. Whatever method is used can also normalize the calculations so that the final value is not affected by nuclear size. This yields a pure measure of roundness unrelated to nuclear area.

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Group components can be used in conjunction with the digital image itself to assess textural properties of important cellular regions. A relatively coarse (global) measure of this kind is total nuclear optical mass, arrived at by summing the densities of all pixels interior to the nuclear perimeter. However, spatial distribution of optical mass within the nucleus is also measurable in various ways. One such technique is to calculate the fraction of total nuclear optical mass present in concentrically located shells of nucleoplasm, starting from the perimeter and proceeding inward towards the geometric center. This type of feature can be expected to yield different distributional data for cells in which there is margination of nuclear chromatin with central karyoplasmic clearing. Such nuclear phenomena are frequently observed when cell vitality is compromised. Further discussion of approaches to image texture is found in (7).

The relationships among geometric and textural cell features may also be analysed. For example, the cellular and nuclear geometric centers can be calculated from the cell and nuclear perimeters. The distance in microns between these two locations is a measure of nuclear centration. Similar statements apply to cell and nuclear center of optical mass. In this case, the digital image values play a role in the computations.

As seen in the examples of cell image analysis given, both components (PATHS, EDGES, PERIMETERS, AND POINTS) and the digital image pixel densities play a role in computation of numerical cell features. Interactive cell image annotation has been included in the Scanning Subsystem to provide identification of important cell geometry independently of computer analysis of the digital image itself. Automatic geometric analysis is possible, desirable, and intended. However, the ability to perform such structural location automatically is dependent upon the densitometric characteristics of the image (6). The detection of objects and boundaries within the cellular image requires usage of edge finding and/or region growing programming technology (5), involves density threshold logic, and frequently requires probablistic interpretation of density patterns in protoplasmic neighborhoods (5).

Densitometric image properties are strongly impacted by the preparatory techniques used in producing the cell populations for scanning- particularly staining. Pixel density is used, as discussed above, in both 1) the location of cellular substructures and 2) in the actual calculation of numerical cell features.

In the development of a cytopathologic assay for water toxicity, it is necessary to assess the utility of various cellular measurements in the discrimination of intoxicated cells from those which are not. This assessment should be performed in advance of any effort to locate cellular infrastructure automatically, to avoid placement of effort into the automatic detection of structures whose numerical properties yield little discrimination. The inclusion of interactive image annotation in the EDIT phase of scanning subsystem makes this possible. Location of important cell structures is dependent

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only upon the operator's ability to see them and not on prior assurance of "machine compatibility" of the preparation.

The numerical features calculated for cells exposed to toxicants will be compared to those for controls treated identically except for titer of test water sample. Feature comparison is, therefore, a relative matter of control values versus experimental, and with normalization of features for variations in staining effects, is rendered fairly independent of absolute densitometric (grey level) properties in the image.

On the other hand, fully automatic location of cellular structures, applicable from cell to cell, slide to slide, experiment to experiment, and possibly stain batch to stain batch, requires tighter control of sample preparation- particularly with respect to fixation and staining. Programs which perform such location can be designed to tolerate a certain amount of variability, particularly if only absolute density levels and not inter-regional contrast or regional texture is affected.

It is best to control preparatory technique as tightly as is practical from the outset, since both feature values and structure location are variably affected. However, it is easier to compensate for such variation when features are evaluated than in structure location. Before an effort is made to automatically locate structures involved in feature calculation, a set of workable features should be determined. This set of features can then be used to clearly delineate the structures for automatic location- and thereby limit efforts in that direction to just those structures needed. In short, feature set before automation and the interactive graphic editing capability of this system permits determination of such a feature set prior to attempt at automation.

When automatic structure location is tried, it is necessary to know how accurate the location procedure is. When, for example, the nuclear border is determined prior to calculation of nuclear area and nuclear textural features, inaccuracies in its delineation will result in feature value error. It is necessary to have some method for assessing "goodness of find."

This type of analysis is frequently omitted in image processing projects under circumstances where it is quite critical. The approach to be used in this project for assessing accuracy in automatic structure location is the measurement of how closely automatically found components overlay interactively traced analogues.

Interactive graphic editing is, therefore, included in this system: 1) to permit feature evaluation before automatic extraction, and 2) to aid in the assessment of automatic location accuracy for those structures required in the calculation of features which prove to be effective discriminators of toxicity.

The organization of graphic data into edit sets, groups, and components which are all named and numbered, combined with only four simple component types arranged in the hierarchy depicted in Figure 8, has been designed to permit a uniform and workable format for performing and recording a virtual and orderly microdissection of the cellular image. The data format can be used, not only when performing this microdissection interactively- as described here, but also when it is done automatically. This approach strongly impacts both software and hardware program complexity and data storage requirements.

Particularly for all-hardware systems implemented for use in production environments, precise data base organization and proper selection of the types and quantity of data captured will markedly affect cost, size, mobility, reliability, resistance to adverse environmental factors, speed, and ease of use. The EDIT phase has been designed to assist in providing workable, cytopathologically oriented algorithms and data for use in such implementations.

In order to promote the validity of statistical inferences made concerning whole populations of cells exposed in culture under identically controlled conditions, provisions for the random selection of cells have been included. Two available approaches can be applied. One is to select cells from a bivariate, uniform distribution over a rectangular area of predetermined length, width, and location on the coverslip. A Second is to select cells from rectangular search zones of independently specifiable dimensions, whose maximum Y and minimum X locations (point in the upper left hand corner of the rectangle as viewed through the microscope) are specified as the random co-ordinates generated. Irrespective of which method is used, random co-ordinates are produced on command to pseudorandom number generating software which is built into the SEARCH phase.

If the coverslip cell population density is not too sparse, the random cell selection method can be used. On the other hand, if the population is sparse, this method might lead to many oil immersion fields which are devoid of cells. This means much wasted motion and time. In this case, the random search zone approach, with selection of all (or a given number of) cells hit during meander search of the zone will more rapidly yield the required number of cells for the coverslip.

In connection with random zone type cell selection, it should be noted that, in addition to the search zone dimensions, the distance between the search lines parallel to the major axis of meander (Figure 5) may be adjusted. In Figure 5 this distance is depicted as delta-X or delta-Y, depending upon which is the major axis of meandering. By setting this distance (in microns) to several multiples of the largest probable test cell's maximum diameter, liklihood of the crosshair intersection hitting the same cell twice during meander search is very small. This facilitates selection of distinct cells and constitutes sampling without replacement. If distinct cells are not required, sampling with replacement can be carried out by selecting all cells, using either random method without regard to previous selections.

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Once cells are selected, their locations can be recorded in a pre-picked list prior to actual scanning. An operator can move to the listed locations and scan the cells. Combined with the parametric and graphic data facilities, the cell selection functions permit supervisory personnel to pre-load the Scanning Subsystem with enough information to exert tight control over data acquisition without being present during the process. The majority of parametric data which does not change can be entered, pre-selection of graphic data edit sets performed, and preselection of cells with recording of their positions accomplished. The system is then turned over to the scan operator for the completion of data acquisition. This frees investigators from frequent interruption in the performance of other project tasks.

TABLES AND FIGURES

TABLE I

SYSTEM SOFTWARE CATEGORIES

CATEGORY NUMBERS	DESCRIPTION	SUPPLIER
1	Disc Operating System (DOS) with Special Equipment Driver Routines	Interdata, Inc., and Project

2	Programming Support:
	    Fortran Compiler
	    Cal Assembler
	    Text Editor
	    Run Time, Subroutine Library
	    Linking Loader
		Interdata, Inc.
		"
		"
		"
		"
		"

3	Scanning Subsystem:
	    Scan Phase Option Selector Program
	    Log-In Program
	    Equipment Initialization Program
	    Search, Locate, and Seek Program
	    Image Edit Program
	    Scan Program
	    Feature Extraction Program
	    Log-Out Program
		Project
		"
		"
		"
		"
		"
		"
		"

4	Utility Subsystem:
	    Scan Data Editing Program
	    Data Directory Editor Program
	    Data Subdirectory Creator Program
	    Source Code Program Filer Program
	    Motorized Stage Test Program
	    Microdensitometer Test Program
		Project
		"
		"
		"
		"
		"

5	Analytic Subsystem:
	    Feature Extraction Division Programs
	    Statistical Division Programs
		Project
		"

6	Display Subsystem:
	    Halftone Image Display Program Division
	    Parametric Data Display Program Division
	    Line Drawing Display Program Division
		Project
		"
		"

7	System Subroutine Library: This library currently consists of more than computer subprograms utilized at various places throughout the entire system. Frequently used functions are programmed only once and a high degree of modularity is maintained.	Project

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FROM PAIR: 1
TO   PAIR: 40

PAIR	XSTEPS	YSTEPS	ORG	STEPLEN	X-MMETERS	Y-MMETERS
1.	72	-275	1	2.00	0.1440	-0.5500
2.	-176	-176	1	2.00	-0.3520	-0.3520
3.	-412	424	1	2.00	-0.8240	0.8480
4.	393	143	1	2.00	0.7860	0.2860
5.	143	-143	1	2.00	0.2860	-0.2860
6.	143	-39	1	2.00	0.2860	-0.0780
7.	378	338	1	2.00	0.7560	0.6760
8.	-123	342	1	2.00	-0.2460	0.6840
9.	-121	-334	1	2.00	-0.2420	-0.6680
10.	110	-488	1	2.00	0.2200	-0.9760
11.	440	-95	1	2.00	0.8800	-0.1900
12.	123	-401	1	2.00	0.2460	-0.8020
13.	462	186	1	2.00	0.9240	0.3720
14.	71	-136	1	2.00	0.1420	-0.2720
15.	164	-316	1	2.00	0.3280	-0.6320
16.	-350	-199	1	2.00	-0.7000	-0.3980
17.	336	316	1	2.00	0.6720	0.6320
18.	-455	-358	1	2.00	-0.9100	-0.7160

PAGE PAUSE- CONTINUE WITH CARRIAGE RETURN

TABLE II

PRINTOUT OF RANDOM COORDINATE DATA GENERATED AND STORED IN SCANNING SUBSYSTEM CONTROL FILE. A MAXIMUM OF 100 SUCH LOCATIONS MAY BE STORED AT ONE TIME.

FROM PAIR: 1
TO   PAIR: 40
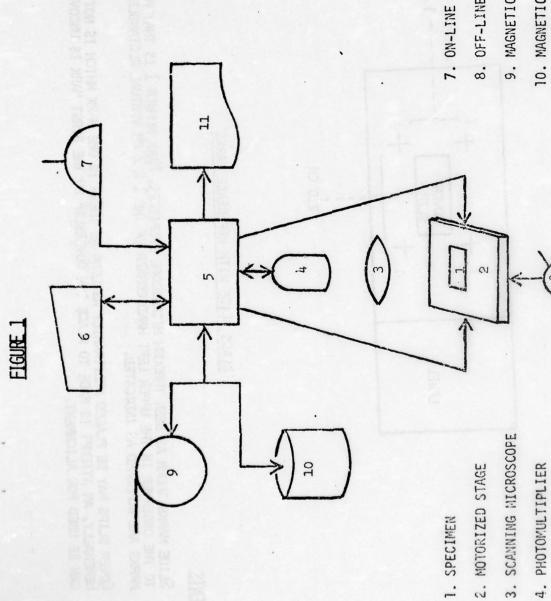
PAIR	XSTEPS	YSTEPS	ORG	STEPLEN	X-MMETERS	Y-MMETERS
19.	-229	-205	1	2.00	-0.4580	-0.4100
20.	313	496	1	2.00	0.6260	0.9920
21.	-118	66	1	2.00	-0.2360	0.1320
22.	351	-124	1	2.00	0.7020	-0.2480
23.	284	60	1	2.00	0.5680	0.1200
24.	-57	-5	1	2.00	-0.1140	-0.0100
25.	114	-75	1	2.00	0.2280	-0.1500
26.	-447	444	1	2.00	-0.8940	0.8880
27.	21	47	1	2.00	0.0420	0.0940
28.	-79	-30	1	2.00	-0.1580	-0.0600
29.	-167	-119	1	2.00	-0.3340	-0.2380
30.	-214	180	1	2.00	-0.4280	0.3600
31.	-478	-281	1	2.00	-0.9560	-0.5620
32.	-17	-470	1	2.00	-0.0340	-0.9400
33.	167	-200	1	2.00	0.3340	-0.4000
34.	-374	-261	1	2.00	-0.7480	-0.5220
35.	120	388	1	2.00	0.2400	0.7760
36.	128	-362	1	2.00	0.2560	-0.7240

PAGE PAUSE- CONTINUE WITH CARRIAGE RETURN

FROM PAIR: 1
TO   PAIR: 40

PAIR	XSTEPS	YSTEPS	ORG	STEPLEN	X-MMETERS	Y-MMETERS
37.	-56	-229	1	2.00	-0.1120	-0.4580
38.	248	79	1	2.00	0.4960	0.1580
39.	4	-206	1	2.00	0.0080	-0.4120
40.	244	-35	1	2.00	0.4880	-0.0700

FIGURE 1

1. SPECIMEN
2. MOTORIZED STAGE
3. SCANNING MICROSCOPE
4. PHOTOMULTIPLIER
5. MINICOMPUTER
6. TELETYPE
7. ON-LINE DIGITAL JOYSTICK
8. OFF-LINE JOYSTICK
9. MAGNETIC TAPE STORAGE
10. MAGNETIC DISC STORAGE
11. PRINTER/PLOTTER
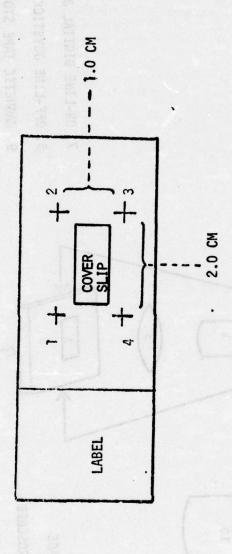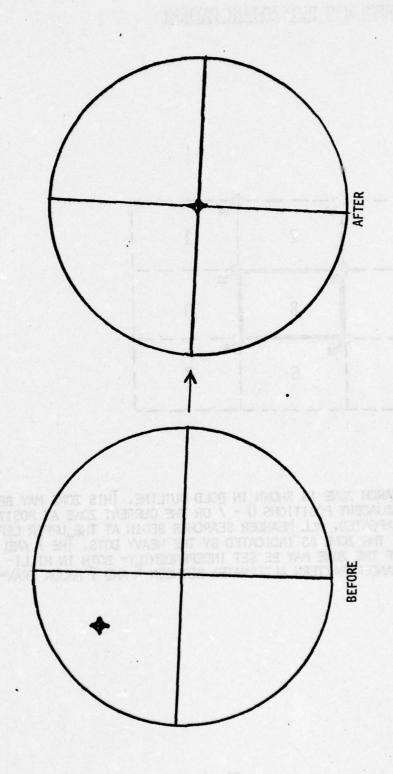
MUST SYSTEM HARDWARE SCHEMATIC DIAGRAM

FIGURE 2

GLASS SLIDE WITH REFERENCE MARKS

COMMENTS

1. SLIDE MARKS SHOWN AS SEEN THROUGH MICROSCOPE EYEPIECES. MARK NUMBER 1 IS THAT WHICH APPEARS TO THE OBSERVER IN THE UPPER LEFT HAND CORNER OF THE 1 x 2 CM VIRTUAL RECTANGLE. THE OTHER MARKS ARE NUMBERED AS INDICATED.

2. COVER SLIPS MAY BE PLACED ARBITRARILY AS LONG AS THERE IS ONE MARK WHICH IS NOT COVERED. GENERALLY, AN ATTEMPT IS MADE TO PLACE THE COVERSLIP SO THE FIRST MARK IS UNCOVERED AND CAN BE USED FOR ALIGNMENT.

FIGURE 3

BEFORE

AFTER
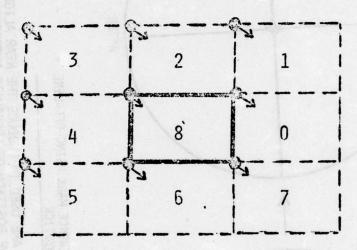
ALIGNMENT ON SLIDE REFERENCE MARK USING OFF-LINE
JOYSTICK

COMMENT: THE MICROSCOPE STAGE THUMBWHEELS ARE USED TO PRODUCE THE NEAR ALIGNMENT SHOWN TO THE LEFT. THE SCENE SHOWS THE RELATIVE POSITION OF CROSSHAIR INTERSECTION AND SLIDE MARK. THE OFF-LINE JOYSTICK IS USED TO PRODUCE EXACT ALIGNMENT AS SHOWN TO THE RIGHT. FINAL JOYSTICK ALIGNMENT IS REQUIRED TO 1) AVOID MISALIGNMENT DUE TO MANUAL PRESSURE EFFECTS AND 2) LOCK STEPING MOTORS INTO GEAR PRIOR TO ON-LINE STAGE MOTION. SINCE THE OFF-LINE STICK MOVES THE STAGE VIA ITS MOTORS, THIS METHOD OF FINAL ALIGNMENT INSURES THAT THE STAGE IS POSITIONED ON STEP BOUNDRIES IN BOTH AXES.

FIGURE 4

SEARCH ZONE DISPLACEMENT PATTERN

3	2	1
4	8	0
5	6	7

COMMENT:

THE CURRENT SEARCH ZONE IS SHOWN IN BOLD OUTLINE. THIS ZONE MAY BE DISPLACED TO ADJACENT POSITIONS 0 - 7 OR THE CURRENT ZONE AT POSITION 8 MAY BE REPEATED. ALL MEANDER SEARCHES BEGIN AT THE UPPER LEFT HAND CORNER OF THE ZONE AS INDICATED BY THE HEAVY DOTS. THE X AND Y DIMENSIONS OF THE ZONE MAY BE SET INDEPENDENTLY- BOTH IN MILLI-METERS. THE MEANDER PATTERN ALTERNATES BETWEEN X AND Y MAJOR TRAVERSE.

FIGURE 5

X-MAJOR MEANDER

YΔ

Y-MAJOR MEANDER

ΔX

DIMENSIONS FOR MEANDER SEARCH

X

Y

SEEK

JOYSTICK SEARCH

AUTOMATIC ZONE SEARCH, JOYSTICK SEARCH, AND SEEKING

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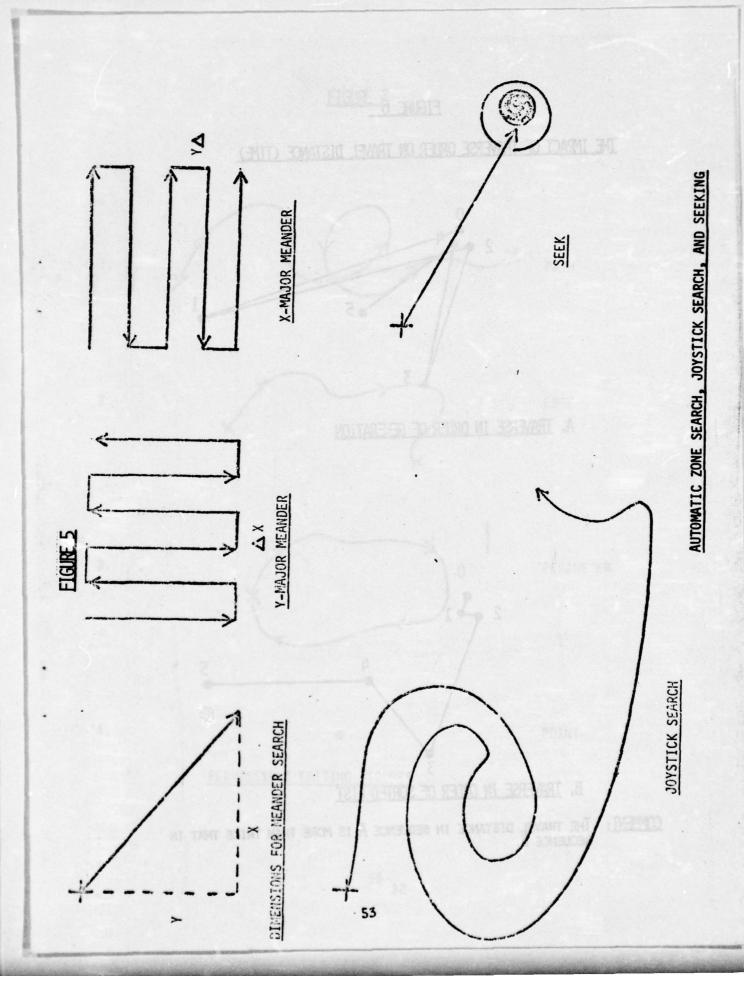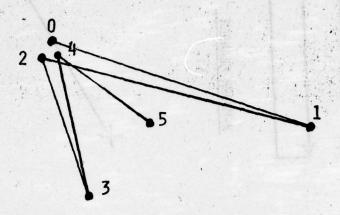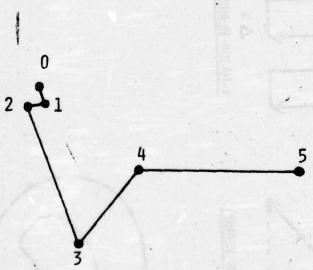
FIGURE 6

THE IMPACT OF TRAVERSE ORDER ON TRAVEL DISTANCE (TIME)
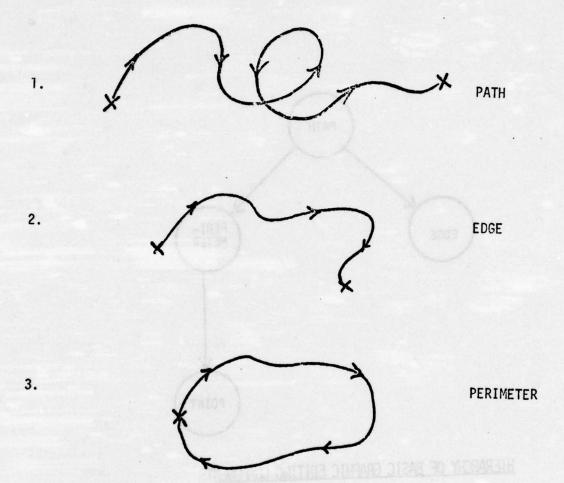
A. TRAVERSE IN ORDER OF GENERATION

B. TRAVERSE IN ORDER OF SORTED LIST
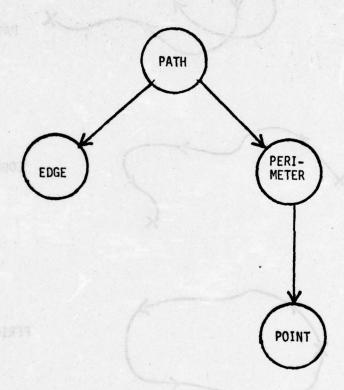
COMMENT: THE TRAVEL DISTANCE IN SEQUENCE A IS MORE THAN TWICE THAT IN SEQUENCE B.

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FIGURE 7

1.   PATH

2.   EDGE

3.   PERIMETER

4.   ●   POINT

PERMISSIBLE EDITING FIGURES
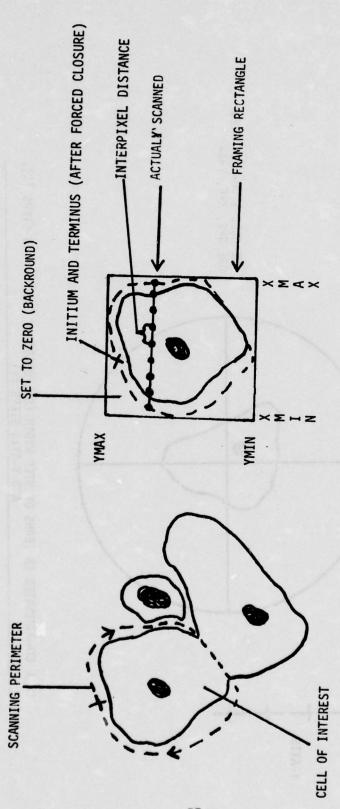
FIGURE 8
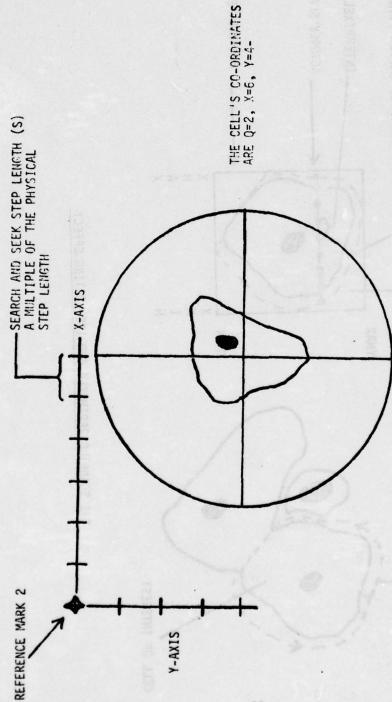
HIERARCHY OF BASIC GRAPHIC EDITING COMPONENTS

COMMENT:

COMPONENT TYPES REPRESENTED BY NODES AT A LOWER LEVEL ARE SPECIAL
INSTANCES OF THE NEXT HIGHER LEVEL PREDECESSOR. PREDECESSOR AND SUC-
CESSOR ARE CONNECTED BY AN ARROW WITH THE PREDECESSOR AT THE TAIL AND
SUCCESSOR AT THE HEAD. SUCCESSORS SATISFY THE DEFINITION OF PREDECESSORS,
BUT MUST ALSO MEET ADDITIONAL DEFINING CRITERIA.

FIGURE 9

INITIUM AND TERMINUS (AFTER FORCED CLOSURE)

INTERPIXEL DISTANCE

ACTUALY SCANNED

FRAMING RECTANGLE

SET TO ZERO (BACKROUND)

YMAX

YMIN

X M A X

X M I N

SCANNING PERIMETER

CELL OF INTEREST

THE SCANNING PERIMETER AND ITS EDITING EFFECT

FIGURE 10

SEARCH AND SEEK STEP LENGTH (S) A MULTIPLE OF THE PHYSICAL STEP LENGTH

X-AXIS

Y-AXIS

REFERENCE MARK 2

THE CELL'S CO-ORDINATES ARE Q=2, Y=6, Y=4-

CELL CO-ORDINATES IN TERMS OF STEP WIDTH (S), QUADRANT (Q), X-AXIS STEPS (X), AND Y-AXIS STEPS (Y)

COMMENT: CELL LOCATION IS IN TERMS OF LOGICAL SEARCH STEP DISTANCE (S). THE CELL LOCATION IS RECORDED AS THE STAGE POSITION RELATIVE TO THE REFERENCE MARK (IN THIS CASE #2), THE CROSSHAIRS INTERSECTION (CURSOR) MUST BE PLACED UNAMBIGUOUSLY ON THE CELL OF INTEREST. THIS SAME SLIDE LOCATION WILL BE USED AS THE ORIGEN-OF-SCAN, BUT THE GRID SPACINGS WILL THEN BE IN TERMS OF INTERPIXEL DISTANCE.

LITERATURE CITED

1. Bahr, G.F., J. A. Boccia, and R. H. Shoemaker, Analysis of Cytotoxic Reactions Produced by MUST-Water Constituents. Annual Progress Report, RCS-MEDDH-288(R1), p 16-22, 1975.

2. Bahr. G. F., J. A. Boccia, and R. H. Shoemaker, Analysis of Ctyotoxic Reactions Produced by MUST-Water Constitutents. Interim Progress Report for period 1 May - 31 August, 1976, Appendix

3. Ibid. (1), p 8.

4. Ibid. (1), p 10.

5. Yakimovsky, Y. Boundry and Object Detection in Real World Images. J. Assoc. Comp. Mach., 23:599-618, 1976.

6. Taylor, J., et al. Development and Evaluation of Nucleus Finding Routines: Thresholding of Cervical Cytology Images. Acta Cytologica, 19:289-298, 1975.

7. Pressman, N. J. Markovian Analysis of Cervial Cell Images. J. Histochem. and Cytochem., 24:138-144, 1976.

8. Freeman, H. Computer Processing of Line Drawing Images. ACM Comp. Surveys, 6:58-97, 1974.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY		1. AGENCY ACCESSION*	2. DATE OF SUMMARY*	REPORT CONTROL SYMBOL
		DA OB 6608	76-10-01	DD-DR&E(AR)636

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8a. DISB'N INSTR'N	8b. SPECIFIC DATA- CONTRACTOR ACCESS	9. LEVEL OF SUM
75-07-01	D. Change	U	U		NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	6.27.73.A	3E762173A819	00	041
b. CONTRIBUTING	6.27.58.A	3A762758A819		
c. CONTRIBUTING	CARDS 114			

11. TITLE (Precede with Security Classification Code)*

(U) Dynamics of Aircraft Accident Victims: Computer Simulation

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
12 07 01	CONT	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
a. DATES/EFFECTIVE: EXPIRATION:		PRECEDING		
b. NUMBER:*		FISCAL 76 T		27
c. TYPE: d. AMOUNT:		YEAR CURRENT		
e. KIND OF AWARD: f. CUM. AMT.				

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
NAME:* Armed Forces Institute of Pathology	NAME:* Armed Forces Institute of Pathology
ADDRESS:* Washington, D. C. 20306	ADDRESS:* Washington, D. C. 20306
	PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
RESPONSIBLE INDIVIDUAL	NAME:* Ballo, J. M., LTC, MC USA
NAME: Hansen, J. L., COL, MC, USA	TELEPHONE: 202-576-3232
TELEPHONE: 202-576-2905	SOCIAL SECURITY ACCOUNT NUMBER:
21. GENERAL USE	ASSOCIATE INVESTIGATORS
	NAME: ROBERT R. McMEEKIN, M. D.
	NAME: LTC, MC/FS, USA

22. KEYWORDS (Precede EACH with Security Classification Code)   (U) Computer Simulation, (U) Aircraft Accidents,
(U) Occupant kinematics, (U) CALSPAN three dimensional program model

23. TECHNICAL OBJECTIVE.* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) Computer simulation of the kinematics of occupants in selected aircraft accidents in order to recreate the sequences of injuries and to determine their specific cause(s). This will lead to an increased understanding of the pathogenesis of such injuries and lead to more effective means of preventing them.

24. (U) Operate the CALSPAN three-dimensional program as a model using selected Aerospace Pathology Division cases as sources of input data. Correlate the actual injuries observed at autopsy with the injuries predicted from the occupant kinematics of the computer simulation.

25. (U) 7507-7609. Eight cases of accidents involving UH1H helicopters with fatalities have been successfully simulated. Changes in DA2397 have improved our ability to simulate such accidents. The body of the report highlights problem areas in the use and interpretation of the CALSPAN Program.

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DD FORM 1 MAR 68 1498   PREVIOUS EDITIONS OF THIS FORM ARE OBSOLETE. DD FORMS 1498A, 1 NOV 68
AND 1498-1, 1 MAR 68 (FOR ARMY USE) ARE OBSOLETE.

ANNUAL PROGRESS REPORT

TITLE PAGE

Project No.   3A762758A819   Title: Dynamics of Aircraft Accident
                                           Victims: Computer Simulation
Task No.   00

Name and Address of Reporting Installation:

Armed Forces Institute of Pathology
Washington, D. C.   20306

Name of Department: Aerospace Pathology Division

Period Covered by Report: 1 July 1975 - 30 September 1976

Professional Authors: J. M. Ballo, LTC, MC, USA
                            R. C. McMeekin, LTC, MC, USA

Report Control Symbol: RCS-MEDDH-288(R1)

Security Classification: Unclassified

BODY OF REPORT

Project No. 3A762758A819   Title: Dynamics of Aircraft Accident
                                          Victims: Computer Simulation
Task No. 00

    Since the last report, dated 4 June 1975, the updated version of
the CALSPAN three-dimensional model has been validated and run. An
improvement in the program is an additional routine which recon-
figures the position of the occupant in order to maintain his orienta-
tion within the vehicle reference system. This allows the components
of the flight path velocity to be decomposed and applied to the vehicle
independently of the yaw, pitch and roll of the vehicle at the time of
impact. This will allow the true effects of inertia to be applied through-
out the crash impulse.

    Field visits to Ft. Rucker have resulted in modification of the
U.S. Army's Form DA 2397. Review of the case material at the AFIP has
shown that although flight path angles and velocities are faithfully
recorded, stopping distances are not so recorded. The addition to the
DA 2397 series of a diagram has facilitated the calculation of crash
forces needed to simulate impact kinematics.

    Eight (8) cases have been successfully simultated: at the present
time we are correlating the observed injuries with those predicted by
the computer program. Preliminary analysis indicates that there is good
agreement in those cases where there is not excessive deformation of the
cabin volume by crushing or intrusion of transmission, motor or rotor
assemblies. The present version of the program does not allow for
deformable panel assemblies.

    Problem areas that have been identified in using the program during
the past year include 1) changing the yaw, pitch and roll angles require
repositioning the occupant; 2) the present seat belt routine is not
interactive and hence not faithful to the operational situation; 3) the
integration routine often fails to converge when the pulse is almost
exclusively vertical. A routine to correct the first problem has been
written and implemented. The CALSPAN Corporation has developed an inter-
active seat belt routine and an improved integrator and this will be
implemented over the next year.

List of Publications

1. "Case for Diagnosis," on Bursting-type Injuries of the Heart, submitted
to Military Medicine, 21 January 1976.

2. "Accident Reconstruction from Analysis of Injuries," submitted and
accepted for publication and presentation at AGARD meeting, 5-9 April 1976,
in Copenhagen, Denmark.

Title: Dynamics of Aircraft Accident Victims: Computer Simulation

3. "Verification of Occupant Injury Patterns as Predicted by Computer Simulation in Fatal Helicopter Accidents," presented at the Tenth Scientific Session of the Joint Committee on Aviation Pathology, 6-12 September 1976 at RAF Halton, United Kingdom.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY		1. AGENCY ACCESSION* DA OB 6605	2. DATE OF SUMMARY* 76 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636		

3. DATE PREV SUM'RY	4. KIND OF SUMMARY	5. SUMMARY SCTY*	6. WORK SECURITY*	7. REGRADING*	8A. DISB'N INSTR'N	8b. SPECIFIC DATA- CONTRACTOR ACCESS	9. LEVEL OF SUM
75 07 01	D. Change	U	U		NL	☒ YES ☐ NO	A. WORK UNIT

10. NO./CODES:*	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	6.47.13.A	1J664713D47	00	001
b. CONTRIBUTING				
c. CONTRIBUTING	CARDS			

11. TITLE (Precede with Security Classification Code)*

(U) Lesions in Animals Fed Enzyme Inactivated Frozen and Irradiated Beef

12. SCIENTIFIC AND TECHNOLOGICAL AREAS*

006500 Food; 014100 Radiobiology

13. START DATE	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY	16. PERFORMANCE METHOD
71 07 01	CONT.	DA	C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
a. DATES/EFFECTIVE:	EXPIRATION:	FISCAL YEAR — PRECEDING 76		32
b. NUMBER:*		7T		
c. TYPE:	d. AMOUNT:	CURRENT		
e. KIND OF AWARD:	f. CUM. AMT.			

19. RESPONSIBLE DOD ORGANIZATION	20. PERFORMING ORGANIZATION
NAME:* Armed Forces Institute of Pathology Washington, D.C. 20306	NAME:* Armed Forces Institute of Pathology Washington, D.C. 20306
ADDRESS:*	ADDRESS:*
	PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)
RESPONSIBLE INDIVIDUAL	NAME:* Casey, H. W., Lt Col, USAF, VC
NAME: Hansen, J. L., COL, MC, USA	TELEPHONE: 202-576-2601
TELEPHONE: 202-576-2905	SOCIAL SECURITY ACCOUNT NUMBER:
21. GENERAL USE	ASSOCIATE INVESTIGATORS NAME: M. A. Stedham, LTC, VC, USA NAME: S. R. Jones, Lt Col, USAF, VC

22. KEYWORDS (Precede EACH with Security Classification Code) (U) Food, (U) Radiation, (U) Sterilization, (U) Beef, (U) Experimental, (U) Laboratory Animals

23. TECHNICAL OBJECTIVE.* 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) To determine the wholesomeness for human consumption of radiation steri-lized beef by studying the pathologic effects, if any, of feeding dogs, rats and mice irradiated beef. Preservation of food is vital to military operations.

24. (U) The U.S. Army is conducting contractual studies on the wholesomeness for human comsumption of radiation sterilized beef. Pathological results obtained from experimental animals will be statistically analyzed and submitted together with other experimental data to the FDA and USDA to establish a regulation permitting unlimited consumption of radiation sterilized beef. The AFIP serves as monitor and reviewer of the pathologic findings in the contractor's experimental and control animals.

25. (U) 7507-7609. During FY 1976 three site visits were made to Industrial Bio-Test Laboratories. During each visit diagnostic terminology was discussed and the contrac-tor's slides were compared with AFIP slides. In addition, necropsy techniques were observed on the first and second visits. The technical preparation of slides by the contractor has remained at a highly acceptable level and the interpretation of histologic changes has been consistent with that of the AFIP with only minor exceptions. The only potential problem is one of accountability for all the animals within the contractor's Quarterly Reports. During the 15 months, 9,500 paraffin blocks were processed and cut into 12,439 slides at the AFIP. Three hundred and forty-one cases were completed and sent to files, and another one hundred and twenty are awaiting microscopic examination.

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*Available to contractors upon originator's approval.

ANNUAL PROGRESS REPORT

TITLE PAGE

Project No. 1J664713D47   Title: Lesions in Animals Fed Enzyme
                                         Inactivated Frozen and
Task No.   00   Irradiated Beef

Name and Address of Reporting Installation:

Armed Forces Institute of Pathology
Washington, D. C. 20306

Name of Department: Veterinary Pathology

Period Covered by Report: 1 July 1975 - 30 September 1976

Professional Authors: H. W. Casey, Lt Col, USAF, VC
                       M. A. Stedham, LTC, VC, USA
                       S. R. Jones, Lt Col, USAF, VC

Report Control Symbol: RCS-MEDDH-288(R1)

Security Classification: Unclassified

BODY OF REPORT

Project No. 1J664713D47

Task No. 00

Title: Lesions in Animals Fed Enzyme Inactivated Frozen and Irradiated Beef

The need for fresh meat to meet the dietary requirements of large numbers of troops in isolated areas where logistics do not permit the use of refrigeration requires the development of a new preservation and sterilization method for meat products. Preservation by radiation offers a practical means of overcoming these logistical problems while maintaining the wholesome and organoleptic properties of fresh meat.

The AFIP serves as monitor and reviewer for the U. S. Army of pathologic findings in experimental and control animals utilized in the present study now under contract with the Industrial Bio-Test Laboratories, Northbrook, Illinois.

All animal feeding tests have been completed so no new material is being generated. The AFIP has just received the paraffin blocks from the 21st Quarterly Report. The results from the 19th Quarterly Report will be coded within 6 weeks and the blocks from the 20th Quarterly Report are being processed.

An additional associate investigator has been added to the project and will shortly be able to apply his time to reducing our lag to less than 3 months.

Three additional studies, each of the same magnitude as the present one, have been started but pathology materials will probably not be received for 12 months.

RESEARCH AND TECHNOLOGY WORK UNIT SUMMARY			1. AGENCY ACCESSION° DA OE 6606	2. DATE OF SUMMARY° 76 10 01	REPORT CONTROL SYMBOL DD-DR&E(AR)636	

3. DATE PREV SUM'RY	4. KIND OF SUMMARY H.Termination	5. SUMMARY SCTY° U	6. WORK SECURITY° U	7. REGRADING°	8a. DISB'N INSTR'N NL	8b. SPECIFIC DATA-CONTRACTOR ACCESS ☑ YES ☐ NO	9. LEVEL OF SUM A. WORK UNIT

10. NO./CODES:°	PROGRAM ELEMENT	PROJECT NUMBER	TASK AREA NUMBER	WORK UNIT NUMBER
a. PRIMARY	6.27.59.A	3A762759A831	00	130
b. CONTRIBUTING				
c. CONTRIBUTING				

11. TITLE (Precede with Security Classification Code)°
(U) Mechanisms of Immunity in <u>Plasmodium berghei</u> Sporozoite Immunized Mice

12. SCIENTIFIC AND TECHNOLOGICAL AREAS°

13. START DATE 75 10 01	14. ESTIMATED COMPLETION DATE	15. FUNDING AGENCY DA	16. PERFORMANCE METHOD C. In-House

17. CONTRACT/GRANT		18. RESOURCES ESTIMATE	a. PROFESSIONAL MAN YRS	b. FUNDS (In thousands)
a. DATES/EFFECTIVE:	EXPIRATION:			
b. NUMBER:°		FISCAL 76		
c. TYPE:	d. AMOUNT:	YEAR CURRENT 7T		45.0
e. KIND OF AWARD:	f. CUM. AMT.			

19. RESPONSIBLE DOD ORGANIZATION		20. PERFORMING ORGANIZATION	
NAME:° Armed Forces Institute of Pathology ADDRESS:° Washington, D. C. 20306		NAME:° Armed Forces Institute of Pathology ADDRESS:° Washington, D. C. 20306	
		PRINCIPAL INVESTIGATOR (Furnish SSAN if U.S. Academic Institution)	
RESPONSIBLE INDIVIDUAL		NAME:° Hockmeyer, W. T., CPT, MSC, USA	
NAME: Hansen, J. L., COL, MC, USA		TELEPHONE:	
TELEPHONE: 202-576-2905		SOCIAL SECURITY ACCOUNT NUMBER:	
21. GENERAL USE		ASSOCIATE INVESTIGATORS	
		NAME: I. Schneider, Ph.D.	
		NAME: S. M. Phillips, M.D.	

22. KEYWORDS (Precede EACH with Security Classification Code)
(U) Mice   (U) Immunity, (U) <u>Plasmodium</u> <u>berghei</u>, (U) Sporozoite,

23. TECHNICAL OBJECTIVE.° 24. APPROACH, 25. PROGRESS (Furnish individual paragraphs identified by number. Precede text of each with Security Classification Code.)

23. (U) This study was undertaken to determine the role of immune serum and immune cells in the adoptive transfer of sporozoite-induced immunity to malaria.

24. (U) Irradiated <u>Plasmodium berghei</u> sporozoites administered to BALB/c mice produce complete protection to challenge with live sporozoites. Serum or spleen cells from immune donors were transferred to recipients which were then challenged with live sporozoites to assay the degree of tranferred protection.

25. (U) 7510-7609. The investigation of sporozoite-induced immunity to malaria in mice has led to several significant results. Using Balb/c mice, an initial immunization with 75,000 irradiated sporozoites gave complete protection against a challenge dose of 10,000 live sporozoites. Circumsporozoite precipitin antibody was present in sera from immune mice and in high titer from mice boosted with 10,000 irradiated sporozoites. Immune serum given to non-immune recipients afforded no protection. Immune cells given intravenously to irradiated mice followed by a boost with irradiated sporozoites gave 100% protection. Preliminary studies using this adoptive cell-transfer system have shown that the recognition phase of immunity is T-cell dependent.

*Available to contractors upon originator's approval.

DD FORM 1498
1 MAR 68

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ANNUAL PROGRESS REPORT

TITLE PAGE

Project No.   3A762759A831   Title: Mechanisms of Immunity in
                                      <u>Plasmodium berghei</u> Sporozoite
Task No.   00   Immunized Mice

Name and Address of Reporting Installation:

    Armed Forces Institute of Pathology
    Washington, D. C.   20306

Name of Department: Microbiology & Immunobiology
                              Pathology Division

Period Covered by Report: 1 July 1975 - 30 September 1976

Professional Author: W. T. Hockmeyer, CPT, MSC, USA

Report Control Symbol: RCS-MEDDH-288(R1)

Security Classification: Unclassified

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BODY OF REPORT

Project No.   3A762759A831   Title: Mechanisms of Immunity in
                                            Plasmodium berghei Sporozoite
Task No.   00   Immunized Mice

Malaria is a significant cause of morbidity and mortality in both
civilian and military populations in the tropics. Although prophylaxis
and treatment with drugs and vector control programs have been partially
successful, malaria remains a major problem. The potential of protecting
people with an anti-malarial vaccine depends on our learning more about
the mechanisms of the immune response to malaria. The present study was
designed to determine the mechanism of sporozoite-induced malaria in a
rodent malaria model.

Irradiated sporozoites, when used to immunize man or mice, have
induced complete protection against a subsequent sporozoite challenge.
Although attempts to characterize host defense mechanisms has implicated
humoral aspects of the immune system, our understanding of these mechanisms
is incomplete.

In both man and rodents, repeated exposure to irradiated sporozoites
results in the development of anti-sporozoite antibodies. These sera
exhibit a sporozoite neutralizing activity (SNA), destroying sporozoite
infectivity and produce a circumsporozoite precipitate (CSP) reaction.
Passive transfer of these antisera to normal recipients results in the
number of expected exo-erythrocytic forms, but ultimately all recipients
develop a parasitemia and die.

The role of cell-mediated immunity in sporozoite-induced immunity
has been less extensively studied because initial attempts at adoptive
transfer of immunity with sensitized spleen or peritoneal exudate cells
were not successful. evidence of cell-mediated immunity is based on the
fact that passive transfer of immune serum does not protect recipient
animals and that, under certain experimental conditions, mice are
protected in the absence of detectible CSP and SNA activity.

Immunity to malaria was studied in an experimental model using
Balb/c mice and Plasmodium berghei sporozoites. The mice, obtained
from Jackson Laboratories, were admitted to experiments at 8-10 weeks
of age. Sporozoites were isolated from the salivary glands of Anopheles
stephensi 14-18 days after a blood meal from infected Syrian Golden
Hamsters. The sporozoite preparations used for immunization received
10 krads in ^{60}Co gamma source (Gamma cell-C).

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Mice were immunized to malaria with irradiated sporozoites intravenously, and all received an initial injection of 7.5 x 10^4 organisms. Depending on the experiment, animals were either used after the initial injection or boosted one or more times with a 1 x 10^4 sporzoites. The standard immunization schedule was one initial injection followed by four boosters seven days apart.

Cells for adoptive transfer procedures were obtained either from peritoneal exudates or spleens of immune donors. Peritoneal exudates were induced by injecting 3 ml of light mineral oil three or five days before cells were collected. Mice were killed by cervical dislocation and peritoneal exudate cells were collected by washing the peritoneal cavity with cold, buffered L15 media and collecting the cells with a sterile pipet after gentle massage. Spleens were removed from the animals under sterile conditions and minced with fine scissors and gently pressed through a 60 gauge mesh into the harvesting medium. The cells were filtered through sterile gauze to remove clumps and slowly drawn through a 26 gauge needle to insure a single cell suspension. The suspensions from several aniamls were pooled, washed three times, and suspended in L15 medium. Cell viability was assessed by trypan blue staining and the cells were counted on a Coulter counter after the red cells were destroyed by isotonic ammonium chloride.

Suspensions of spleen cells were fractionated using nylon wool columns into adherent cell populations. Both fractions were assays for surface immunoglobulin (Ig) using fluorescein-conjugated rabbit antisera. The non-adherent cells were less than 10% Ig positive and will be referred to as T-cells. The adherent cells were over 90% surface Ig positive and will be referred to as B-cells. Following fractionation, cells were washed, resuspended in TC199 medium and counted.

Test cell suspensions, consisting of either non-immune control, immune unfractionated, immune-T cells or immune-B cells, and in varying quantities were transferred to recipient mice intravenously. The recipients had been exposed to 600 rads in a ^{60}Co gamma source eight hours prior to cell transfer. These animals were then boosted 24 hours later with 10^4 sporozoites intravenously.

In order to evaluate protective immunity, the recipient animals were challenged intravenously with 10^4 sporozoites. This dose produced infections in 98% of control animals, which were all lethal. Three days after challenge Giemsa-stained blood films were examined daily for ten days to determine the length of the prepatent period. Those animals with prolonged prepatent periods compared to non-immunized controls were considered to be partially immune. On certain groups of animals parasitemias were monitored until death. Cumulative mortaligy and parasitemia was monitored on all animals at 24 hour intervals. Immunized animals which did not develop an infection upon challenge were considered to be immune.

Anti-sporozoite antibodies were measured in immunized nude, hetero-zygous nude, and homozygous control groups which received either a single injection of 7.5×10^4 sporozoites or a single injection plus four boosters of 1×10^4 sporozoites and which were bled from the retro-orbital plexus seven days after the last injection. The sera from mice within a group were pooled and tested for CSP and SNA activity according to standard methods.

In one experiment the role of macrophages in the immune response was evaluated by treating recipient mice with macrophage-blockading-agent (Carrageenan-Cg.) for 14 days prior to challenge with sporozoites accord-ing to a protocol known to significantly reduce macrophage function.

The ability of various quantities of immune unfractionated cells to convey protection was investigated. In the initial studies with the cell-transfer system one "spleen-equivalent" or about 150 million cells were transferred to each irradiate recipient. Actually, far less cells are required to transfer immunity as shown in Table 1.

Table 1. Quantity of immune cells required to transfer protection.

Cells Transferred $\times 10^6$		Positive Parasitemia/total mice
Non-immune	200	8/8
	100	4/5
	40	10/10
	20	7/9
Immune	200	0/8
	100	0/10
	40	0/16
	20	0/31
	10	1/21
	5	2/18
	2.5	0/8
	1	1/13

The results of the cell fractionation experiments are shown in Table 2. It is clear that immunity is transferred with T-cells and not with B-cells.

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Table 2. Type of immune cells required to transfer protection.

Cell Source	No. of Cells (mil)	Positive Parasitemia
Normal	2.5	9/9
	1.0	6/6
Immune	2.5	0/8
	1.0	1/13
T - immune	2.5	0/8
	1.0	1/6
	0.5	2/7
B - immune	2.5	2/2
	1.0	5/6

When carregeenan-treated mice served as the recipients in cell transfer experiments, the results were identical to those experiments using untreated recipients as shown in Table 3.

Table 3. Effect of carregeenan on transfer of immunity.

Cells Transferred		Recipient	Parasitemia
Normal	1.0 mil.	untreated	6/6
Normal	1.0 mil.	Cg. treated	6/6
Immune	1.0 mil.	untreated	0/6
Immune	1.0 mil.	Cg.treated	0/6
T - immune	1.0 mil.	untreated	1/6
T - immune	1.0 mil.	Cg. treated	0/6

The duration of immunity transferred was studied by delaying challenge until four weeks following cell-transfer. In this experiment, mice which received 1.0 mil. immune cells, or immune T-cells, or Immune B-cells were all protected. The protection observed in the B-cell transferred group was explained by the opportunity for proliferation of a small number of T-cells that contaminate the B-cells transferred.

These results demonstrate that the mechanism of sporozoite-induced immunity requires T lymphocytes and is completely operative in the absence of either B lymphocyte or macrophage function. The most natural explanation for the mechanism is either direct T-cell killing of sporozoites, or elaboration of a soluable factor (lymphokine) by T-cells which is lethal for sporozoites.

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The induction of sporozoite-induced immunity, including the induction of CSP and SNA activity is T-cell dependent. None of the nude mice, even those multiply boosted, demonstrated a protective response upon challenge or any CSP or SNA antibody activity. The heterozygous and homozygous mice were protected with when immunized singly or when boosted. Antibody, interms of CSP and SNA activity, does not appear to be a primary defense mechanism as protection was consistently shown in the absence of CSP and SNA activity. This is not to say that antibody plays no role, but only that other mechanisms in and of themselves are insufficient to confer protection. Adoptive transfer experiments indicate that the primary mechanism in sporozoite immunity on the efferent end is T-cell, as transfer of sensitized B-cells conferred no protection in non-immune recipients, while transfer of T-cells resulted in complete protection. The actual mechanism(s) by which T-cells confer protection is open to further investigation.

It is recommended that additional studies using this excellent cell-transfer model be performed to determine the details of the mechanism of immunity to sporozoite-induced malaria. In particular, since there is an indication that T-cells have a major role, perhaps as killer cells, a series of in vitro experiments could be done using immune cells and sporozoite suspensions. Such studies might provide useful information not only for the study of immunity to malaria but for the whole field of immunity to parasitic disease.

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